HERG and STAT1 Interactions in Estrogen Receptor Positive and Estrogen Receptor Negative Human Breast Cancers

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Abstract

The human ether-a-go-go-related gene (HERG) potassium channel, a known regulator of cell proliferation, is overexpressed in several cancer cell lines. Despite its importance, there have not been many studies regarding the mechanism by which it contributes to aberrant proliferation of cancer cells. In this study, we identified a novel estrogen signalling pathway that plays a role in regulating cell proliferation in estrogen receptor positive breast cancer cells. We provide the initial characterization of this signal transduction pathway which results in up-regulation of HERG channels and increased proliferation of estrogen receptor positive breast cancer cell lines.

Using biochemical and confocal microscopy imaging, we revealed that there is a protein-protein interaction between HERG and Signal Transducers and Activators of Transcription 1 (STAT1) in breast cancer cell lines that express estrogen receptors (ER+) (MCF-7, T47D) and in those that lack ERs (ER-) (MDA-MB-231, BT-20). After estrogen treatment (E2, 10μM), only the ER+ human breast cancer cell lines showed increased co-precipitation of HERG and STAT1 and higher levels of subcellular colocalization, and these effects were prevented by pharmacological blockers of ERs (ICI 182 780, 5nM) or STAT1 (fludarabine, 50μM). Furthermore, we demonstrated that the enhanced STAT1 and HERG interaction induced by estrogen is important for upregulation of the HERG channel surface expression. Consistent with our prediction that HERG surface expression plays a critical role in cancer cell proliferation, proliferation assays revealed a marked upregulation of ER+ cancer cell proliferation after E2 stimulation, and this was prevented by ICI, fludarabine and the HERG blocker E4031 (10μM). Together, these results suggest that there is an estrogen receptor mediated signalling pathway involving a physical complex between STAT1 and HERG channels, and this proposed pathway may be an attractive strategy for anti-cancer therapeutic targeting of ER+ human breast tumors.

Next, since it is known that HERG contains multiple tyrosine residues whose phosphorylation could be increased after estrogen receptor stimulation, we hypothesized that these phosphorylated tyrosine residues could serve as binding sites of the SH2 domain of STAT1. We have used FR-peptide, a 28 amino acid peptide mimetic of STAT1 SH2 domain, to disrupt interaction between HERG and STAT1. Our biochemistry and imaging data demonstrated that FR-peptide did disrupt the HERG-STAT1 interaction, and this cell-permeable peptide did prevent the estrogen-induced upregulation of HERG channel and cancer cell proliferation of ER+ breast cancer cells. Finally, we also demonstrated that the HERG-STAT1 interaction was specific to breast cancer cells, as similar biochemical studies failed to show physical interactions in normal brain and cardiac tissue.

Together, this study reveals a novel estrogen receptor signalling pathway which enhances the HERG channel surface expression and its contribution to cancer cell proliferation in ER+ human breast cancer cell lines. The identification of the FR-peptide to occlude HERG-STAT1 interaction and prevent HERG surface expression and cancer cell proliferation, may form the basis for a rational design of anti-cancer therapeutics. The regulatory factors involved in the regulation of endogenous HERG-STAT1 interaction in ER- cell line remains to be established.

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List of Abbreviations

AP1	Activator Protein 1
CK	Cytokeratin
ERE	Estrogen Response Element
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
ER+	Estrogen Receptor positive
ER-	Estrogen Receptor negative
E1	Estrone
E2	Estradiol
E3	Estriol
FBS	Fetal Bovine Serum
HERG	Human *Ether-à-go-go* Related Gene
HER2	Human Epidermal growth factor Receptor 2
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IGF-1	Insulin like Growth Factor-1
LQT2	Long QT syndrome type 2
MAPK	Mitogen-Activated Protein Kinase
miR	microRNA
PBS	Phosphate Buffer Saline
PI3K	Phosphoinositide 3-Kinase
PR	Progesterone Receptor
PTK	Protein Tyrosine Kinase

pTyr	Phosphorylated tyrosine
PVDF	Polyvinylidene Fluoride
SERD	Selective Estrogen Receptor Degrader
SERM	Selective Estrogen Receptor Modulator
SH2	Src Homology 2
STAT	Signal Transducer and Activator of Transcription
SP1	Specificity Protein 1
TBST	Tris Buffer Saline with Tween 20

Dedication

I dedicate this thesis to my loving family, Tae-Heung, Jung-Suk and Jae-Yong.

1. GENERAL INTRODUCTION

1.1 Breast Cancer

Breast cancer is the most common type of malignancy and the leading cause of cancer death among females worldwide [1]. Amongst all types of cancer diagnosed in women, its incidence accounts for about 23 % and has 14 % mortality rate [2]. Though the etiology of this devastating disease is still unclear, known risk factors are age, ethnicity, high mammographic density, increased alcohol consumption, genetic factors and hormone replacement therapy [3].

1.1.1 Different types of breast cancer

Breast tumours are diverse and their molecular variation results in different responsiveness to treatments [4]. Based on gene expression patterns, there are mainly 4 different subtypes of breast cancers, including luminal A, luminal B, basal and HER2 [5]. The luminal and basal subtypes of breast cancer are distinguished by expression of cytokeratins [6]. Both of the luminal subtypes which arise from the endothelial luminal cells lining the mammary ducts express high levels of luminal cytokeratins (CKs), including CK 7/8, 18 and 19 [6]. Luminal A subtype is characterized by expression of estrogen receptor (ER+) and progesterone receptor (PR+), lack of human epidermal growth factor receptor 2 (HER2-), and low expression of Ki67, which is a cellular marker for proliferation [5]. Luminal A tumours grow slowly compared to other tumour subtypes, have the best prognosis, and represent the most common subtype, accounting for about 50% of all breast tumour occurrence [7]. In contrast, luminal B subtypes account for about 20% of all cases, and are ER+, PR+, and HER2+ with high expression of Ki67 [5]. Both of the luminal subtypes depend on estrogen signalling for cell division and proliferation. Therefore, anti-estrogen adjuvant therapy using Selective Estrogen Receptor

Modulators (SERMs), aromatase inhibitors and Selective Estrogen Receptor Downregulators (SERDs) are effective in the treatment of ER+ tumours.

Basal subtype, which arises from the outer basal cell layer of human breast, is distinguished by high expression of cytokeratins 5, 14 and 16 [6]. Basal subtype accounts for about 15 to 20 % of the entire breast cancer incidents. The majority of basal subtype cancer lacks expression of ER, PR and HER2; therefore, this subtype is often called triple-negative breast cancer. Since basal subtype breast cancer does not express the key hormone receptors or HER2, surgical intervention is the only treatment known so far [6]. Basal subtype tumours are more aggressive and have a poorer prognosis compared to the luminal subtype tumours [8]. Since the basal subtype tumours are ER-negative and HER2-negative, they cannot be treated with hormone therapy or Herceptin (an antibody which directly blocks HER2). Current therapeutic intervention of basal type tumours include surgery, radiation therapy and chemotherapy, however, epidermal growth factor receptor and androgen receptor are potential targets for future therapies [8].

HER2 subtype accounts for about 15 % of all breast cancer incidence [7] and it is characterized by ER negative, PR negative and overexpression of HER2, a receptor-type tyrosine kinase [5]. Anti-HER2 drugs such as Herceptin can be used to treat HER2 type breast cancer.

1.1.2 Estrogen and Breast Cancer

Estrogen is a steroid hormone that plays a role in growth, development and maintenance of female sexual characteristics and reproduction. There are three different types of estrogens produced in women: estrone (E1), estradiol (E2), and estriol (E3). Among these different types, estradiol is the most active form of estrogens. The role of estrogen in the development of breast tumours has been well established. The sizes of breast tumours in breast cancer patients were

observed to be changing during menstrual cycle due to the changes in estrogen production [9]. Furthermore, surgically removing ovaries from breast cancer patients resulted in dramatic remission of the breast tumour [10]. Thus, oophorectomy was a popular intervention for breast cancer before anti-estrogen adjuvant therapy was introduced.

The plasma level of estrogens, especially E2, significantly goes down in postmenopausal women [11] and this increases risk for postmenopausal women to develop a number of health conditions including osteoporosis [12], heart disease [13] and depression [14]. Therefore, postmenopausal women sometimes choose to receive hormone replacement therapy to alleviate such postmenopausal health conditions. However, the hormone replacement therapy is associated with increased risk of developing breast cancer [3].

1.1.3 Estrogen signalling and breast cancer

The estrogen receptor (ER) is a ligand-regulated transcription factor that belongs to the nuclear receptor superfamily [15]. There are two subtypes of ERs, ERα and ERβ. Both subtypes are expressed in various tissues including breast, ovaries, prostate, bone, liver, heart, thyroid and brain tissue [16]. However, the expression level of the two ERs varies from tissue to tissue and the expression ratio has been shown to be functionally important [17]. In the normal breast tissue, ERβ was found to be the predominant form of ER. ERβ was found in about 80-85% of the normal breast cells, whereas, ERα was only found in 7-10% of the normal breast cells. However, different expression levels of ERs were found in cancerous breast tissue suggesting different roles for the ERs [18-22]. The expression level of ERα was found to be increased in breast cancer suggesting that ERα is a positive regulator of cell proliferation [18-20]. In contrast, reduced expression level of ERβ was observed in ER positive MCF-7 cells and in a number of breast tumours suggesting anti-proliferative and tumour suppressing roles of ERβ [21].

ERs become activated when estrogen binds to them. Even though all three types of estrogens bind to estrogen receptors (ERs), their binding affinity and binding preference differ [23]. E2 has the highest affinity for ERα of all the different subtypes of estrogens and has equal binding affinity for ERα and ERβ. E1 has higher affinity for ERα over ERβ, whereas, E3 has higher affinity for ERβ over ERα [23]. The activated ERs dimerize and translocate to the nucleus where they can directly interact with their target gene promoters through estrogen response elements (EREs) [24]. The activated ERs can also interact with their target genes indirectly through other DNA-bound transcription factors, such as members of the activating protein-1 (AP-1), specificity proteins -1 (SP1) and Signal Transducer and Activator of Transcription 1 (STAT1) [24-26].

ERα regulates various genes including pS2 [27] and cyclin D1 [28] that are involved in proliferation of cells [29]. The pS2 gene is an estrogen-responsive gene that is only expressed in breast cancer cells but not in normal breast cells [30, 31]. In MCF-7 cells, ectopic expression of pS2 was shown to be highly associated with increased proliferation [32]. ERα regulation of cyclin D1 demonstrates a clear mechanism for E2-induced cell proliferation. Cyclins are proteins which play a crucial role in controlling the cell cycle progression, and they are responsible for G1 to S phase transition in breast epithelial cells [33]. Cyclin D1 is a type of cyclin which is responsive to E2 signalling [28]. Overexpression of cyclin D1 has been shown to increase the probability of developing breast tumours, whereas deficiency in cyclin D1 has been shown to arrest mammary gland development [34].

Estrogen can also regulate cell proliferation though extra-nuclear signalling (non-genomic). The majority of the ERs are found in the nucleus but there are some ERs found in the cytoplasm [35]. E2 binding to ERs localized in cytoplasm triggers several signal transduction

pathways [36, 37]. Several of the downstream targets of non-genomic ERα activation include growth factor receptors, such as epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 (IGF-1) receptor [38]. ERα activation of the IGF-1 gene has been shown to have a proliferative effect in breast cancer cells [39]. An extra-nuclear action of E2 also activates the *src*/p21ras/MAPK pathway in breast cancer cells [40]. Upon E2 binding, ERα immediately and transiently interacts with *src* and as a result stimulates *src* activity [41]. *Src* activation subsequently increases active p21/ras protein, which in turn stimulates MAP-kinase activity [40]. MAPK cascades propagate and amplify signals involved in cell growth, proliferation and differentiation and thus abnormalities in MAPK signalling is highly associated with the development and progression of breast cancer [42]. The phosphatidylinositol-3-kinase (PI3K) pathway is another downstream signalling pathway of non-genomic activation of ER known to participate in cell growth, proliferation and differentiation [43]. It has been shown that ERα activation activates PI3K by binding to p85 regulatory subunit of PI3K, thereby increasing estrogen-induced cell proliferation in ER positive breast cancer [44].

1.2 HERG Potassium Ion Channel

1.2.1 Cancer and Potassium Channels

Recently, potassium channels have been recognized as important players in cancer pathology as they contribute to cancer initiation and progression by regulating cell proliferation [45-47]. Inhibition of potassium channels has been reported to decrease the proliferation of various cell types [45]. Regulation of cell proliferation by potassium channels can be explained by their capability to regulate the membrane potential [48, 49]. Membrane potential is a net electrical charge created by the difference in ionic concentration between the intracellular and extracellular environment. Many studies have shown that the cells with little or no mitotic activity, such as muscle cells and neurons, have a hyperpolarized resting potential, whereas highly proliferating cells such as cancer cells have depolarized membrane potentials compared to non-proliferating cells [48, 50-53]. Furthermore, membrane potential changes throughout the cell cycle progression and the membrane potential of the cells going through M phase are shown to be depolarized [54].

1.2.2 Structure and Role of HERG

The human *ether-à-go-go* related gene (HERG) encodes the pore-forming alpha subunit of the voltage-gated potassium channel, which is composed of 6 transmembrane-spanning alpha helices [55, 56]. Each HERG subunit has N- and C-terminal regions that are located intracellularly and may play critical roles in regulatory pathways [57].

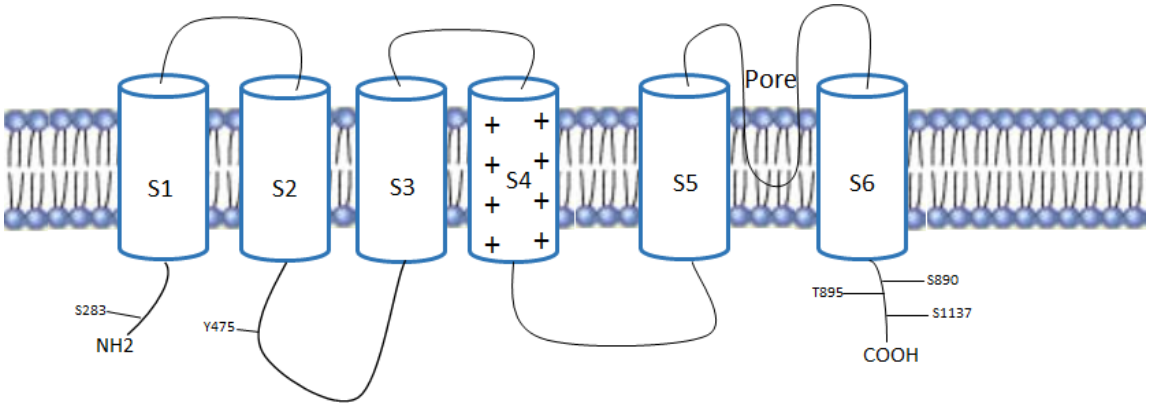


Figure 1.1 Topology of HERG Channel. Diagram of an α-subunit of the HERG channel. Each α-subunit is composed of six transmembrane domains (S1-S6) with cytoplasmic NH2 terminus and COOH terminus. S4 region is positively charged and it acts as a voltage sensor. S5 and S6 membrane domains form the pore of the channel. Known binding sites for PKA (S283, S890, T895 and S1137) and *src* (Y475) are shown on the diagram.

The role of HERG is best understood in the heart. HERG plays a crucial role in regulation of a repolarizing current in the heart [58-60]. This current, termed I_{kir}, terminates the plateau phase of the action potential (AP) [55, 56]. HERG has unique gating kinetics that are responsible for its role in regulating repolarization. HERG has slow activation and deactivation processes, but its voltage-dependent inactivation and recovery from inactivation processes are extremely fast [61]. Therefore, upon repolarization, HERG potassium conductance increases as recovery from inactivation occurs much faster than channel deactivation. These distinct biophysical characteristics of HERG channels distinguish HERG from other voltage-gated potassium channels that are normally activated (opened or increased conductance) by depolarization. In contrast to other voltage-gated potassium channels, over-expression of HERG channels is associated with increased resting membrane potentials [62, 63]. Any factor causing a disturbance in HERG function such as loss of function mutations [59] or use of HERG blocking

drugs [64] can induce long QT syndrome type 2 (LQT 2), which is a lethal ventricular arrhythmia characterized by irregular heart beat [56].

HERG is not only expressed in cardiac tissues but also in many non-cardiac tissues, including neuronal tissues [65], pancreatic tissues [66], smooth muscle tissues [67] and several cancerous tissues [68]. In the non-cardiac cells, HERG channel participates in setting the resting membrane potential [69]. The role of HERG channels in cancer cell proliferation warrants further elucidation.

1.2.3 Regulation of HERG

The HERG channel (HERG1) consists of 1159 amino acids (approximately 127 kDa core protein), whereas HERG2 and HERG3 are 994 and 958 amino acids, respectively [70]. The immature (core-glycosylated) and mature (poly-glycosylated) forms of HERG are found in different subcellular locations. The immature HERG refers to a core-glycosylated monomer with a molecular mass of approximately 135 kDa and the mature HERG refers to a fully glycosylated HERG, with a molecular mass of 155 kDa. The HERG protein is synthesized as core-glycosylated monomers in the endoplasmic reticulum, and are then assembled into tetramers [71]. Once they are transferred to Golgi apparatus, they become fully glycosylated. Subsequently, the mature HERG gets transported to the plasma membrane where it is functional and contributes potassium membrane currents [71]. This post-translational modification of HERG via glycosylation is critical for trafficking efficiency and stability of HERG channel on the cell surface. Mutation of N-linked glycosylation site (N629) is shown to impair HERG trafficking to the plasma membrane [72].

In addition to glycosylation, HERG has numerous consensus phosphorylation sites for different kinases including serine/threonine protein kinases [73-76] and protein-tyrosine kinases

(PTKs) [77, 78]. Many studies have revealed that phosphorylation of the HERG channel is crucial for regulating its function. Cayabyab et al. showed that *src* tyrosine kinase (*src*) can increase HERG current by shifting the voltage dependence of activation to more negative potentials and slowing down the deactivation [77]. The same study showed that inhibiting *src* activity using PTK inhibitors, such as genistein and herbimycin A, causes a reduction in HERG current, whereas activating endogenous *src* with *src*-activating peptide increases the current [77]. Furthermore, phosphorylation of HERG does not only participate in regulation of HERG function but it is also critical for HERG's protein binding capacity. Phosphorylation can facilitate protein-protein interactions, thereby coordinating various cellular signalling pathways.

Recently, transcriptional regulation of HERG overexpression has been revealed. It has been reported that the promoter region of the HERG gene contains binding sites for many oncoproteins and tumour suppressors including NKx3.1, SP1 and NF-kappaB, which have been shown to regulate HERG transcription in cancer [79]. Preventing SP1 and NF-kappaB binding to the promoter region of HERG decreased HERG promoter activity, whereas inhibiting NKx3.1 activity increased HERG transcription in SK-BR3 breast cancer cell line [79]. More recently, post-transcriptional regulation of HERG by microRNAs, including miR-328, miR-22 and miR-23a, and miR-133b (for HERG1) [80-82] and miR-224 for HERG2 [83] have been shown to decrease HERG expression and reduce cancer cell proliferation. Other forms of HERG regulation, including HERG DNA methylation, require further elucidation [84]. However, post-translational modification of HERG is by far the most studied aspect of HERG regulation [62]. In this thesis, I will characterize the potential post-translational modification of HERG by STAT1 (see Section 1.3), although possible transcriptional regulation of HERG by STAT1 remains to be established.

1.2.2 Role of HERG in cancer

The majority of previous studies on HERG was done on its function in the heart, however in recent years, special interest was given to the role of HERG in cancer research as it is found to be overexpressed in many types of cancer [63, 85]. Analyzing the differential expression patterns of HERG gives a clue to the channel's role in cell proliferation. HERG is transiently expressed at the developmental stage of muscle cells and quail neural crest-derived neurons, but its expression is replaced by classic inward rectifier-like potassium currents at later stages [86, 87]. Interestingly, neural crest cells that go through malignant transformation re-express high levels of HERG [86, 87]. Furthermore, developmental changes of erg1 current in mouse heart was observed [88, 89]. The erg1 current was shown to be the predominant repolarizing current in fetal mouse heart, but it disappeared in adult mouse heart [88, 89]. These observations reveal that HERG expression is important for proliferating cells because its overexpression results in depolarized membrane potentials. The depolarized membrane potential of various tumour cells may be explained by overexpression of HERG [63]. Together, the differential patterns of HERG expression revealed HERG's important role in cell cycle progression and cell proliferation.

Indeed, many studies have provided more direct links between the role of HERG and cell proliferation in normal and cancerous cells [63, 87, 90]. It has been reported that replacing HERG with other inward rectifier-like potassium channels induced hyperpolarized membrane potential, and subsequently inhibited cell proliferation [91, 92]. Furthermore, recurrent expression of HERG channel was observed during transformation and differentiation of highly proliferating neuroblastoma cell line [63]. It has been reported that several tumour cells including leukemic [63, 93] and neuroblastoma [94] cancerous cells overexpress the full length HERG protein as well as the N-terminal truncated isoform HERG1b [94]. The N-terminal truncated

version is 819 amino acids in length (approximately 90kDa), which is significantly shorter than the immature and mature forms of full-length HERG (135-155kDa) [94]. The N-terminus of HERG regulates deactivation kinetics and N-terminal deletion results in much faster deactivation [63]. HERG1b can produce a functional HERG current but its deactivation kinetic is about 5-fold faster than the normal HERG current [94, 95]. As a result, HERG1b isoform is shown to reduce the HERG current conductance. Interestingly, HERG and HERG1b are shown to co-assemble in various tissues including the heart tissue [96], brain tissue [65] and cancerous tissues [94]. Moreover, HERG1 and HERG1b are differentially expressed during cell cycle phases [94]. The full length HERG is up-regulated during G1 phase, whereas, the N-truncated isoform is up-regulated during S phase [94].

1.2.3 Therapeutic value of HERG channel in cancer

Overexpression and functional relevance of HERG channel in cancer makes the HERG channel a potential target for anti-cancer drugs. HERG has a large pore cavity that can accommodate different types of drugs [97]. Indeed, there has been a number of HERG blocking drugs including the anti-histamine astemizole and the class III anti-arrhythmic agent E4031 that have been shown to prevent cell proliferation [98-100]. However, HERG blocking drugs are associated with a high risk of cardiotoxicity [71]. Now, all new drugs being synthesized have to go through HERG screening to ensure they do not block the HERG channel. Therefore, more studies need to be done to better understand HERG regulation, especially in cancerous cells, in order to identify novel signalling mechanisms that can be specifically targeted for cancer therapeutics.

1.3 STAT1

1.3.1 STAT1

Signal Transducer and Activator of Transcription 1 (STAT1) is a transcription factor that belongs to the STAT protein family [101]. As its name suggests, STAT1 transduces signals from transmembrane receptors into the nucleus where it activates the transcription of target genes. STAT1 is best understood for its role in the immune system in regulating the inflammatory processes during pathogen infections [102]. However, STAT1 also modulates many cellular processes including cell proliferation and cell apoptosis. Therefore, the expression level of STAT1 is a powerful prognostic tool for different types of cancers, including metastatic melanoma [103], colorectal cancer [104], pancreatic cancer [105] and breast cancer [106].

1.3.2 Structure and STAT1 signalling

STAT1 is composed of six domains: the DNA-binding, N-terminal, coiled-coil, linker, *src* homology 2 (SH2) and transactivation domains (Fig. 1). The functions of these domains have been well studied. The DNA-binding domain binds to DNA and it also plays a role in nuclear translocation. The linker domain is involved in transcriptional activation by facilitating STAT1 binding to gamma-activated sites (GAS), which is crucial for a gene activation [107, 108]. The N-terminal domain forms a site for dimerization and tetramerization which regulates the nuclear translocation of STAT1 [109]. The coiled-coil domain facilitates STAT1 interaction with interferon regulatory factors (IRF) family proteins [110, 111]. The SH2 domain, which is very highly conserved in the STAT family binds to phosphorylated tyrosine residues in the intracellular domains of cytokine receptors including the interferon gamma (IFN-γ) receptor and

the interferon alpha (IFN-α) receptor [112, 113]. The SH2 domains are also critical for STAT1 activation and phospho-dimer formation [111].

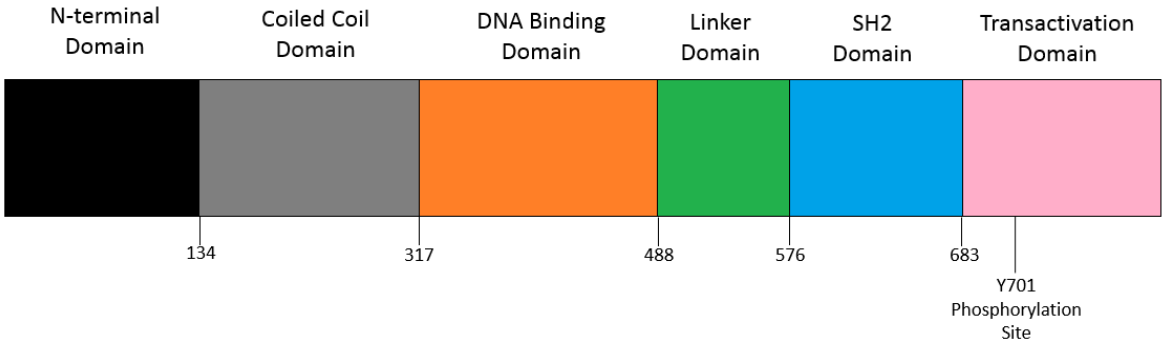


Figure 1.2 Schematic structure of STAT1. The figure shows 6 domains of STAT1 and the regulatory C-terminal tyrosine residue (Y701) responsible for STAT1 dimerization. It is unclear whether the SH2 domain of STAT1 mediates interaction with HERG channels.

Upon stimulation by cytokines or growth factors, STAT1 becomes tyrosine phosphorylated by JAK [114] and *src* kinases [115] at the tyrosine 701 residue, which is located at the C-terminus. Subsequently, it dimerizes with other STAT1 or other members of STAT family. The dimerization is achieved through reciprocal binding of phosphorylated tyrosine residue of STAT to the SH2 domain of the binding partner. The dimers then translocate into the nucleus by binding to importin-alpha [116] where it regulates target gene expression [117]. Although the STAT1 SH2 domain binds to various tyrosine residues involved in signal transduction, it remains to be established whether STAT1 SH2 domain also participate in the subcellular localization and functional regulation of membrane-localized ion channels, including HERG channels.

1.3.3 STAT1 in cancer

The role of STAT1 in cancer is controversial. Traditionally, STAT1 is considered as a tumour suppressor. The anti-cancer function of STAT1 in cancer is best illustrated in STAT1-deficient mouse studies, which showed that gene deletion increased susceptibility to tumorigenesis [118]. An increased spontaneous ER+ mammary tumour formation was observed in STAT1-/- mice in two studies suggesting the anti-proliferative role of STAT1 [118, 119]. Other studies have also shown that STAT1 is transcriptionally regulating other transcription factors, such as c-myc and CDK6, which play a role in cell cycle progression [120-122].

In contrast, there is growing evidence that STAT1 is involved in the development of tumours. An elevation of STAT1 activity was reported in breast tissue compared to normal breast tissue [123]. Furthermore, an *in vitro* study conducted on breast cancer cells suggested that STAT1 activity is creating an immunosuppressive tumour microenvironment by inhibiting the anti-tumor T-cells [124].

1.3.4 HERG, STAT1 and ER

As previously mentioned, estrogen can regulate cell proliferation in cancerous cells by binding to cytoplasmic ERs. Non-genomic activation of ERs has a downstream target which is known to regulate HERG current, namely the *src* tyrosine kinase. HERG current has been reported to be regulated by tyrosine phosphorylation [77, 125]. Thus, aberrant estrogen signalling in breast cancer may reinforce HERG function by activating *src* in ER+ breast cancer. The *src* family kinases also activate STAT proteins which are also known to regulate proliferation of cancer cells [126-128]. Therefore, there may be a crosstalk between HERG and STAT1 mediated by activated *src* in ER positive breast cancer.

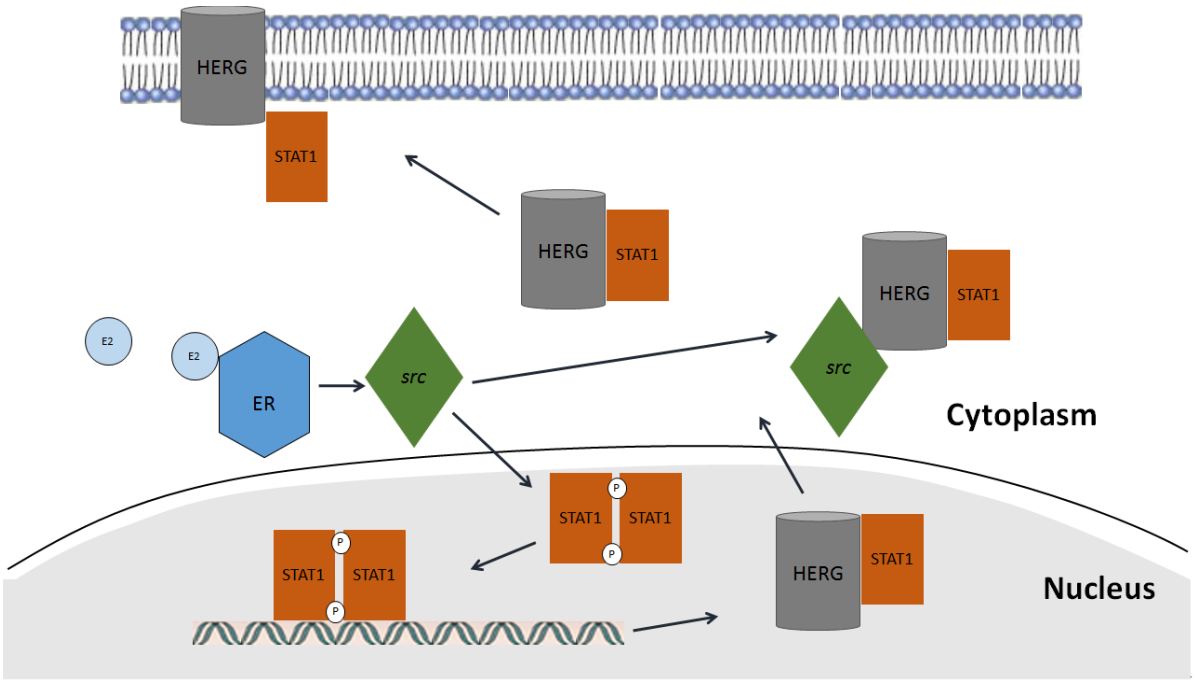


Figure 1.3 Proposed model for estrogen-induced overexpression of HERG channels in estrogen receptor-positive human breast cancer cell lines. Estrogen stimulation leads to increase in HERG and STAT1 interaction through *src* activity. The interaction between HERG and STAT1 leads to increased expression of HERG channels on the surface, as a result, enhancing breast cancer cell growth. *Src*-mediated activation of STAT1 could also lead to dimerization and translocation to the nucleus where it turns on HERG gene transcription. However, this study will only focus on the post-translational regulation of HERG by protein-protein interaction with STAT1.

1.4 Research Objectives and Hypotheses

The main objective of this thesis is to examine the interaction of HERG and STAT1 in response to estrogen stimulation in human breast cancer cells.

Specific Aims:

1. To investigate HERG and STAT1 interactions in breast cancer.

HERG has tyrosine residues that can be phosphorylated by cytosolic tyrosine kinases and STAT1 has a SH2 domain that could potentially bind to phosphorylated tyrosine residues in HERG. We hypothesize that increased *src* activity results in an endogenous HERG and STAT1 interaction in breast cancer cells, and this interaction can be potentiated by estrogen stimulation.

2. To examine the effect of estrogen on HERG and STAT1 interaction.

Estrogen, a key regulator of cell proliferation in breast cancer, activates *src* tyrosine kinase by binding to ERs [129]. This activation of *src* tyrosine kinase can phosphorylate tyrosine residues on the HERG channel [77]. In breast cancer where there is aberrant signalling of estrogen, *src* may be constitutively active to phosphorylate its target substrates, including HERG and STAT1. Therefore, we hypothesize that estrogen will increase the HERG and STAT1 interaction.

3. To examine the functional role of estrogen stimulation on HERG and STAT1 interactions.

HERG channels contribute to produce more depolarized membrane potential, therefore, regulation of HERG is important in cancer physiology. It has been shown that tyrosine phosphorylation is important for HERG function [77, 125]. Therefore, if estrogen increases HERG function through *src* and STAT1 binding, we hypothesize that estrogen stimulation will increase cell proliferation of ER positive breast cancer cells.

4. To examine if a mimetic STAT1 SH2 domain (FR-peptide) exhibits an anti-proliferative effect in breast cancer cells by disrupting the STAT1-HERG interaction.

If HERG and STAT1 binding is mediated by SH2 domain and pTyr motifs, we hypothesize that FR-peptide will prevent estrogen-induced cell proliferation by disrupting HERG and STAT1 interaction.

2. GENERAL METHODS

2.1 Cell culture

Four different human breast cancer cell lines were utilized in this study: MCF-7 (ER+), MDA-MB-231 (ER-), BT-20 (ER-) and T47D (ER+). MCF-7 cells were grown in MEM medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (antibiotic treatment), 1% sodium pyruvate, and 0.16% human insulin. MDA-MB-231 cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. BT-20 cells were grown in EMEM medium (Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin. Finally, T47D cells were grown in RPMI 1640 medium that was supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.2 units/ml bovine insulin. All cell lines were cultured at 37°C in a humidified 5% CO_2 cell incubator.

2.2 Cell proliferation assay

Assessment of cell proliferation was performed using the WST-1 colorimetric dye (Clontech Laboratories, Inc., Mountain View, CA). Cells (4×10^3 per well) were seeded into 96-well plates and cultured for 48h. Then they were treated with the estrogen receptor antagonist ICI 182780 (5nM, Tocris, Bristol, UK), the HERG-selective potassium channel blocker E-4031 dihydrochloride (10μM, Tocris), the STAT1 inhibitor Fludarabine (50μM, Tocris), src tyrosine kinase inhibitors herbimycin A (10μM) and PD 166 285 (100nM), or FR-peptide (30μM) for 1 hour followed by the β-Estradiol treatment (3μM, Sigma-Aldrich, St. Louis, MO). The cells were then incubated with WST-1 for 4 hours at 37°C prior to taking measurement at different time

points; after incubation, the absorbance (at 450nm) was measured against a reference wavelength of 620nm at 4h and 48h. Each treatment was done in triplicates.

2.3 Biochemistry

When MCF-7, T47D, BT-20 and MDA-MB-231 breast cancer cell lines were seeded in T25 plates and have reached 70% confluency, cells were then incubated in new culture medium (7ml) with pharmacologic agents Fludarabine (50μM), herbimycin A (10μM), PD 166 285 (100nM), E4031 and FR-peptide (30μM) for 30 mins to 1h, followed by treatment with estrogen (3μM) for 48h. After treatments, cells were washed with ice-cold phosphate-buffered saline (PBS). Cells were then incubated with 1 mg/ml NHS-SS-Biotin (Thermo Scientific, Waltham, MA) at 4°C for 45min and the reaction was quenched with a quenching glycine buffer (Glycine and Tris, PH 7.6). Following the biotinylation, cells were homogenized with 500μL of lysis buffer containing protease inhibitors and 1% NP-40 detergent (Sigma). After determining the protein concentrations using Bradford assay, equal amounts of protein lysates (200-500μg) were diluted in lysis buffer (Tris, NaCl, EDTA, NaF, PMSF, Aprotinin, Pepstatin A, Peupeptin and NA3Vo4), and biotinylated proteins were incubated overnight with the streptavidin beads (Thermo Scientific). The beads were then washed 2-3 times on the next day with lysis buffer containing 0.1% NP-40 detergent (Sigma). The proteins were eluted by adding 50μl of 2X Laemmli sample buffer (Bio-Rad), and the samples were boiled at 95°C for 5 min and ran on 10% SDS-PAGE gels.

Immunoprecipitation was performed to examine interactions between HERG and STAT1, HERG and *src*, or STAT1 and *src* by incubating 500μg of protein extract from cell lysates with 25μL of 50% slurry protein A/G agarose beads (Sigma) for an hour at 4°C. After this pre-clearing

stage, the agarose beads were removed by pulse spinning at 6000rpm for 5s, and the supernatant was subsequently reacted with an immunoprecipitating antibody against HERG (Alomone, Jerusalem Isreal), STAT1 (Santa Cruz Biotechnologies, Santa Cruz, CA), or *src* (Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4°C. After overnight incubation of lysates with an anti-HERG (5µg, polyclonal rabbit), anti-STAT1 (5µg, polyclonal rabbit) or anti-*src* antibody (5 µg polyclonal rabbit), 50µl of 50% slurry agarose beads were added and incubated for 4 hours then collected by pulse spins. The beads were washed four times with wash buffer (solubilization buffer containing 0.1% NP-40). Then proteins were eluted by adding 50µl of 2X Laemmli sample buffer (Bio-Rad), and the samples were boiled at 95°C for 5min. The samples were ran on 10% polyacrylamide gel and then electrotransferred to polyvinylidene fluoride membrane (PVDF, Millipore, Billerica, MA). After blocking with 5% non-fat milk in TBST (Tris buffered saline containing Tris and NaCl with 10% tween 20) for 1 hour at room temperature or overnight at 4°C, the membranes were incubated with primary antibody in 5% non-fat milk in TBST containing 0.025% sodium azide overnight at 4°C. The PVDF membranes were washed with TBST for 15 minutes four times, and then incubated with a rabbit horseradish peroxidase-conjugated secondary antibody against IgG (1:1000; Santa Cruz) in 5% non-fat milk blocking solution. After four 15 minute washes with TBST, proteins were visualized using enhanced chemiluminescence (Santa Cruz). Densitometry analysis was performed using ImageJ software (see below).

2.4 Immunohistochemistry, immunocytochemistry and confocal imaging microscopy

Pre-fixed, paraffin-embedded 4µm slices of healthy and pathology certified cancerous breast tissue (Prosci Inc. U.S.A) were first de-paraffinized with xylene, then permeabilized with 0.3% Triton X-100 and blocked with 5% BSA in PBST (Phosphate buffered saline containing

NaCl, KCl, Na2HPO4 and KH2PO4 with 10% tween 20). Then the tissues were incubated first with anti-HERG (goat, Santa Cruz, 1:100) and either anti-STAT1 (rabbit, Santa Cruz, 1:100) or anti-*src* (rabbit, Santa Cruz, 1:100) overnight at 4°C and then in appropriate secondary antibodies (1:1000) for an hour. After subsequent nuclear labeling with Hoechst stain, the cells and tissues were mounted on slides.

The four cell lines were grown on coverslips. For immunocytochemistry, cells were treated with Fludarabine (50µM) for 2 hours before estrogen treatment (3µM). After 48 hours of estrogen treatments, cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% TritonX-100, and blocked with PBS containing 5% bovine serum albumin for an hour at room temperature (BSA, Sigma). HERG, STAT1 and *src* were labeled by overnight co-incubation (at 4°C) with goat anti-HERG and either rabbit anti-STAT1 or rabbit anti-*src* diluted at 1:150 in blocking buffer. This was followed by brief washes (three times; 10 minutes each) and subsequent incubation with Alexa Fluor 555-conjugated or Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) at 1:1000 for 1h at room temperature. Then the cells were labeled with Hoechst (Sigma). Lastly, the coverslips were mounted on newly cleaned slides using Prolong Gold Antifade Reagent (Invitrogen, Carlsbad, CA).

Images were taken with a Zeiss LSM 700 laser scanning confocal fluorescence microscope (Carl Zeiss, Oberkochen, Germany), and Particle Analysis and Intensity Correlation Analysis were performed using plug-ins from the WCIF (Wright Cell Imaging Facility) Image J software (public domain, software download from NIH version 1.44f). The degree of colocalization between fluorescent probes was quantified using the Intensity Correlation Analysis plugin in Image J, which calculated the Pearson's correlation coefficients, where values closer to 1 indicated a stronger degree of colocalization between the two labelled proteins. Particle counts were

determined by performing background subtraction of images, and setting threshold detection to 2 SDs above the mean background intensity and particle sizes between 1 μm^2 and infinity. Images were then converted to grey-scale values before applying the Particle Analysis feature of Image J. Statistical analysis used unpaired t-test, and $p<0.05$ was considered significant.

2.5 Preparation of FR-peptide

FR-peptide (>98% purity) was synthesized by Dg Peptide Co., LTD (Hang Zhou, China). The fluorescent labelling of the peptide was done using 5-carboxyfluorescein (Sigma) according to the manufacturer's instructions. The 5-carboxyfluorescein was dissolved in DMSO and the FR-peptide and its scrambled version were incubated with the 5-carboxyfluorescein overnight at 4°C. The labelled FR-peptide and scrambled FR-peptide were purified using the peptide purifying column (Bio-Rad). Non-labelled peptides or 5-carboxyfluorescein reagents alone were used as negative controls for the labelled peptides.

2.6 Data Analysis

Protein densitometry analysis was performed using Quantity One (Bio-Rad) software program. Measured densitometry values of protein bands were normalized to either beta actin or GAPDH. Then the densitometry values for each of the treatment groups were normalized to their control. Statistical significance of results between treatment groups in biochemical, confocal imaging, and proliferation studies were examined using a student's unpaired *t*-test or a one-way ANOVA with a Student Neuman-Keuls post hoc test with a confidence level of $p<0.05$ depending on the number of treatment groups.

3. RESULT: ESTROGEN-INDUCED HERG AND STAT1 INTERACTIONS IN HUMAN BREAST CANCER

3.1 Breast cancer tissue shows both significantly increased expression and co-localization of HERG and STAT1 compared to healthy tissue.

Many studies have reported that HERG and STAT1 are found in breast cancer [79, 90, 98, 118, 130, 131], but it is not yet known whether these proteins are expressed in the same subcellular compartments and whether they exist in a multi-molecular physical complex to regulate cancer cell growth. Here, we first examined the expression level of HERG and STAT1 in cancerous human breast tissue compared to the normal tissue. Using confocal imaging microscopy and particle analysis, we showed that HERG expression (shown in green, Fig. 3.1A) was significantly higher in cancerous breast tissue compared to healthy tissue. Similarly, STAT1 (shown in red, Fig. 3.1B) was also expressed at significantly higher levels in the cancerous tissue compared to healthy tissue. Merged images of HERG and STAT1 (Fig. 3.1C) showed that a high degree of co-localization (shown in yellow) occurred only in the cancerous tissue but not in the healthy breast tissue. Interestingly, HERG and STAT1 appeared to co-localize in the cytosolic and perinuclear compartments. Also, there is colocalization occurring inside nuclear compartments (within Hoechst-stained regions). Since a plasma membrane marker was not included, it is not clear whether colocalization also existed at this region. Taken together, these data show that HERG and STAT1 are both overexpressed in human breast cancerous tissue compared to the normal breast tissue and they exist in a multi-protein complex in different subcellular regions.

Figure 3.1

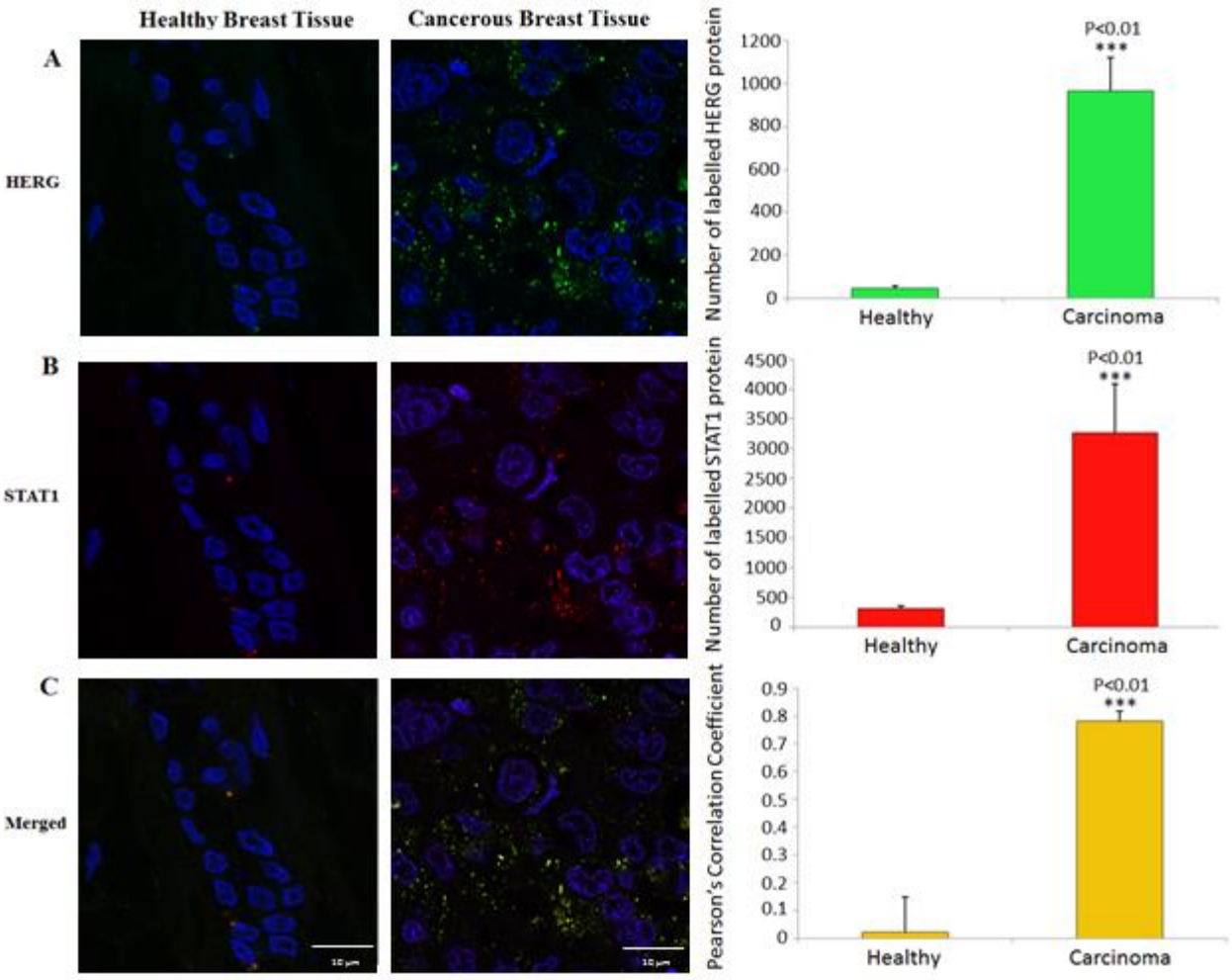


Figure 3.1 Comparison of HERG and STAT1 expression and their colocalization in healthy versus cancerous breast tissue. A. HERG expression denoted by green was significantly greater in cancerous breast tissue than in healthy breast tissue. B. STAT1 expression denoted by red was significantly greater in cancerous breast tissue than in healthy breast tissue. C. The colocalization of HERG and STAT1, denoted by yellow, was comparably higher in cancerous tissue compared to health tissue; values are mean ± SEM; n=7 for healthy and n=5 for cancerous breast tissue slices; ***p<0.01. Blue denotes nuclei in all images and particle analysis was performed using Image J software.

3.2 HERG and STAT1 interact in ER+ and ER- human breast cancer cell lines

To further investigate a potential interaction between HERG and STAT1, we performed co-immunoprecipitation studies of HERG and STAT1 using four different human breast cancer cell lines: the ER+ MCF-7 and T47D and the ER- MDA-MB-231 and BT-20 cell lines. In all four cell lines, immunoprecipitation with a STAT1 antibody showed HERG immunoreactivity in the STAT1 immunoprecipitates (Fig. 3.2 A), indicating that these two proteins interact with each other. Conversely, immunoprecipitation with the HERG antibody and subsequent immunoblotting with STAT1 antibody showed that the HERG immunoprecipitates also contained STAT1, indicating that HERG and STAT1 were in a physical complex together (Fig. 3.2 B). We also confirmed that HERG immunoprecipitates contained *src* (Fig. 3.2 C), which is also contained in STAT1 immunoprecipitates (Fig. 3.2 D).

Figure 3.2

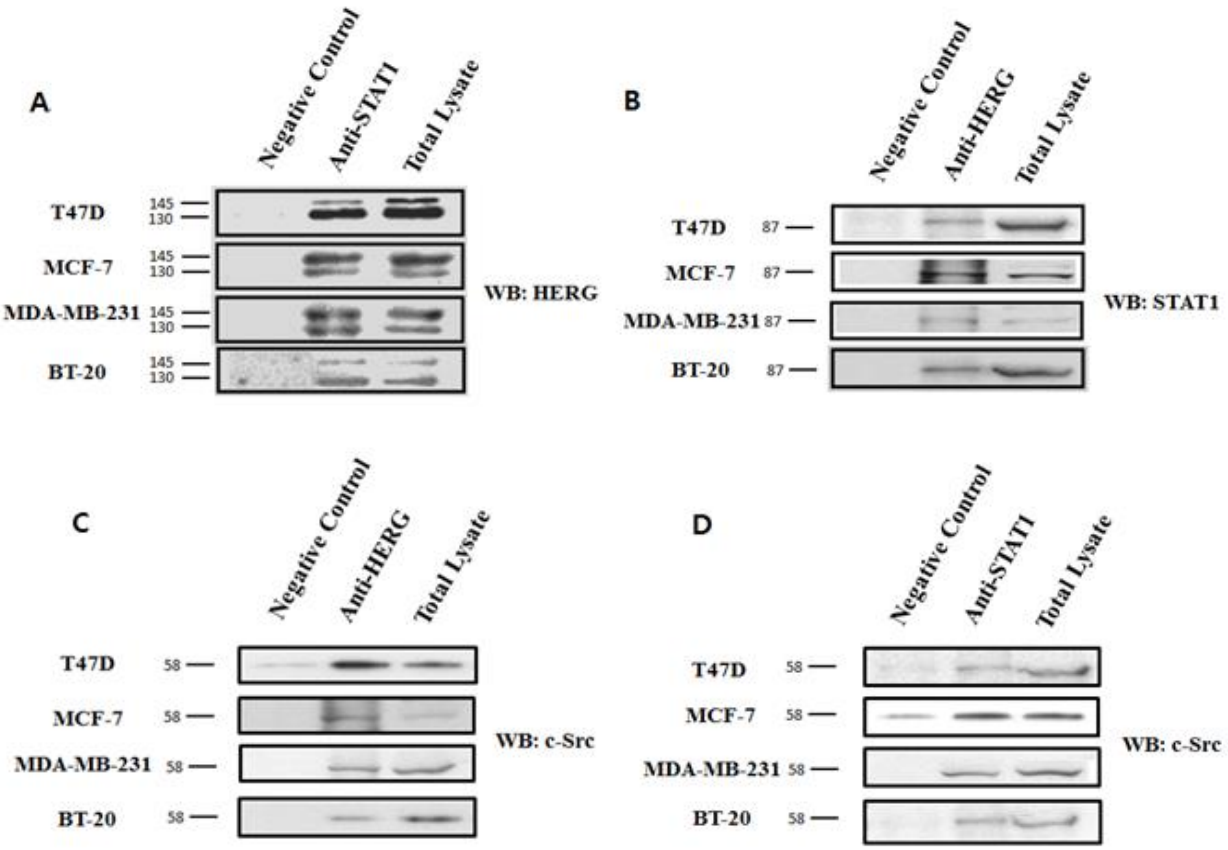
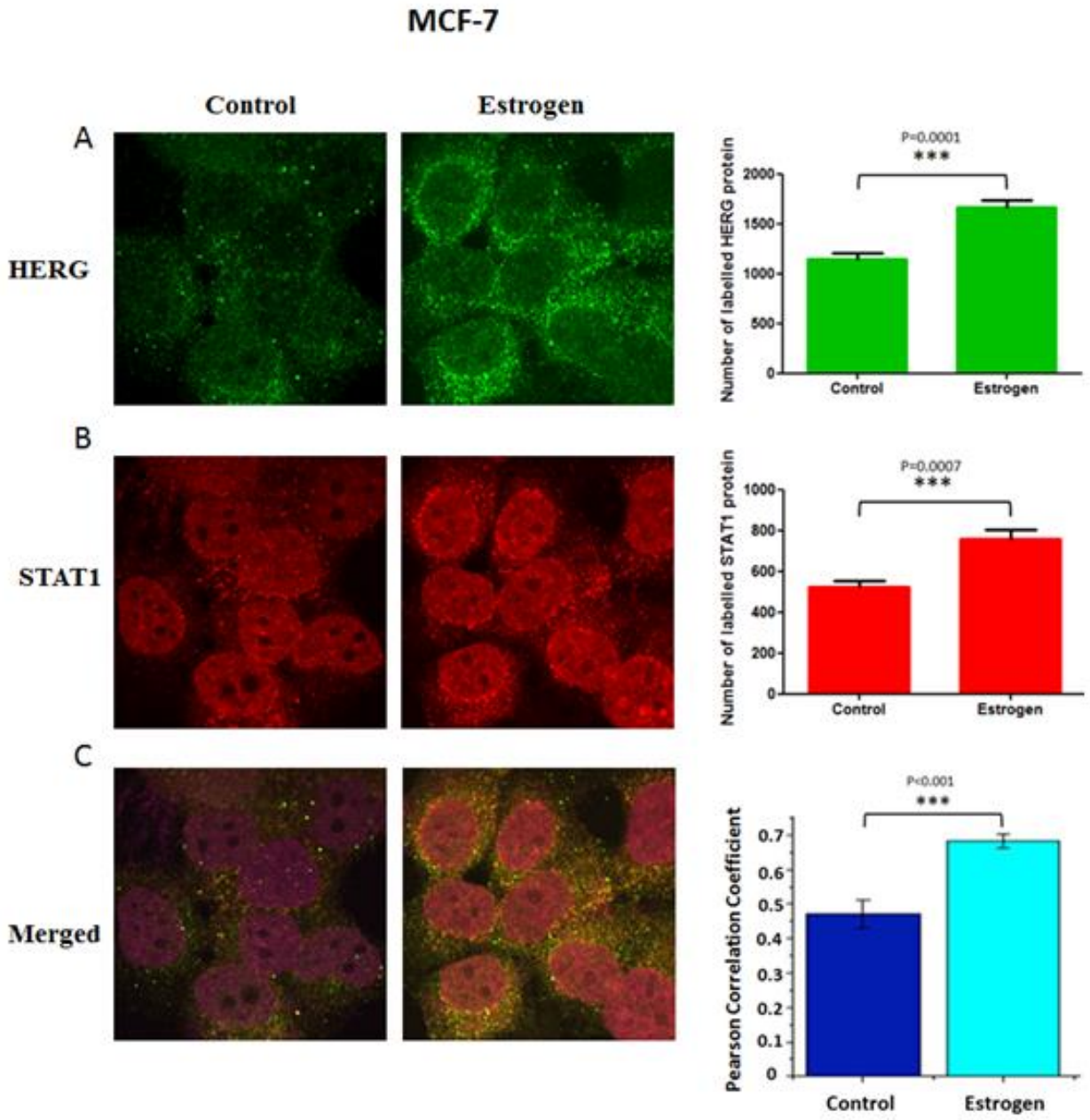


Figure 3.2 Co-immunoprecipitation of HERG and STAT1 in MCF-7 (ER+), T47D (ER+) BT-20 (ER-) and MDA-MB-231 (ER-) cell lines. A. STAT1 immunoprecipitates (lane 2) and positive control lysates (lane 3) showed HERG doublets at near 130 and 150 kDA in all four cell lines. B. HERG immunoprecipitates (lane 2) and positive control lysates (lane 3) showed STAT1 band at near 87 kDA in all cell lines. C. HERG immunoprecipitates (lane2) and positive control (lane 3) showed *src* band at near 58 kDa. D. STAT1 immunoprecipitates (lane2) and positive control (lane 3) showed *src* band at near 59 kDa. Negative controls (lane 1) represent the omission of the immunoprecipitating antibody, and rabbit IgGs were used.

3.3 Estrogen treatment increases STAT1 and HERG co-localization in MCF-7 cells.

Next, having shown that HERG and STAT1 exist in a physical complex, we hypothesized that interaction between the two proteins can be potentially regulated by estrogen receptor stimulation. To test this hypothesis, ER+ MCF-7 cells and ER- MDA-MB-231 cells were treated with estrogen. Endogenous levels of HERG and STAT1 were found in both MCF-7 and MDA-MB-231. In MCF-7 cells, immunofluorescence staining in the presence of estrogen showed increased expression of both HERG and STAT1 compared to the control. Moreover, estrogen induced higher colocalization of HERG and STAT1 in the cytoplasmic and nuclear distributions, as shown by the yellow pixels in the merged images. The same experiment was done on MDA-MB-231 cells. The relative increase in the degree of colocalization of HERG and STAT1 in MDA-MB-231 cells was smaller compared to that observed in MCF-7 (compare Figs. 3C and 3F), but this modest increase was significantly greater compared to the control untreated cells (Fig. 3F).

Figure 3.3


MCF-7
Control
Estrogen
A
HERG
B
STAT1
C
Merged
P=0.0001
***
Number of labelled HERG protein
2000
1500
1000
500
0
Control
Estrogen
P=0.0007
***
Number of labelled STAT1 protein
1000
800
600
400
200
0
Control
Estrogen
P<0.001
***
Pearson Correlation Coefficient
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Estrogen

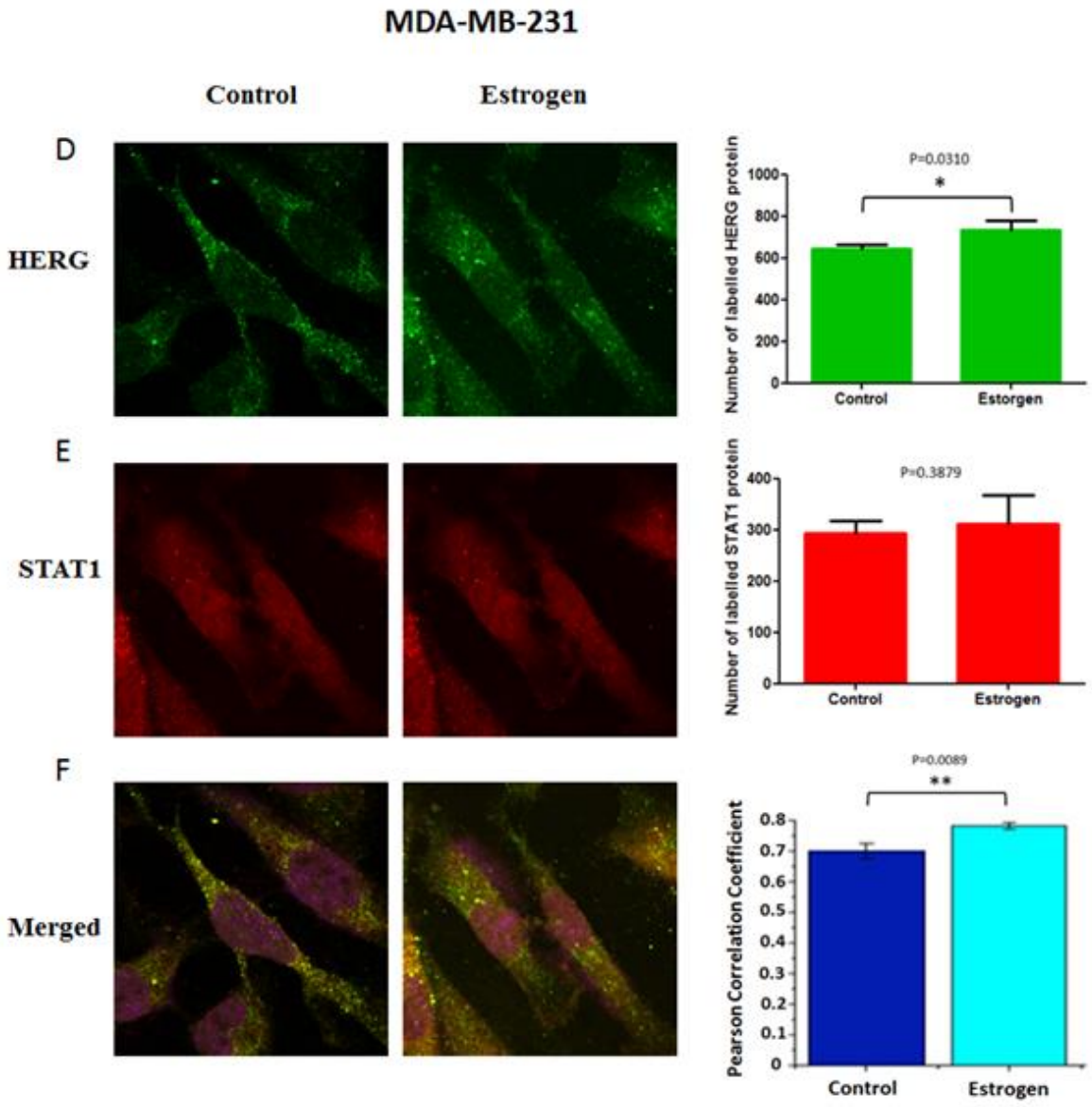


Figure 3.3 Confocal images showing the effect of estrogen on HERG and STAT1 colocalization in MCF-7 and MDA-MB-231 cell lines. A-C: Estrogen treatment (3μM) (right panels) increased HERG (green) and STAT1 (red) expression, resulting in increased colocalization (yellow punctate) between HERG and STAT1 compared to controls (left panels) in MCF-7 cells. The quantified data shows significant increase in the degree of colocalization in MCF-7 cells when they are treated with estrogen (C); Values are mean ± SEM, and n=9 for the control and n=10 for the estrogen treated group; ***p<0.001 by paired t-test. D-F: Estrogen treatment also increased colocalization of HERG and STAT1 in MDA-MB-231 cells (F); however, the magnitude of increase was not as high as it is in MCF-7 cells (F vs C). The Pearson correlation coefficient values (mean ± SEM; n=7) are increased in MCF-7 (P value is 0.0089 vs. P value <0.001 for MCF-7). **p<0.01;*p<0.05.

3.4 Estrogen treatment induces increased surface expression of HERG channels in ER+ human breast cancer cells: Role of STAT1

Having shown that an endogenous interaction between HERG and STAT1 exists in ER+ and ER- human breast cancer cell lines, we tested the hypothesis that HERG expression and interaction with STAT1 was regulated by ER stimulation. To investigate the possible potentiating effects of estrogen on HERG expression and also the effect of estrogen-induced HERG and STAT1 interactions in human breast cancer cell lines, cell surface biotinylation experiments were performed. We hypothesized that HERG surface upregulation can be increased by estrogen stimulation and subsequent STAT1 activation. Biotinylation results showed that fludarabine, a selective STAT1 inhibitor, indeed blocked the estrogen-induced upregulation of HERG surface expression (Fig. 3.4 A lanes 3 vs. 2). However, it did not change the surface expression of HERG in the MCF-7 cell line on its own (Fig. 3.4 A lane 4), suggesting that the estrogen-induced HERG surface upregulation is dependent on STAT1 activation but other regulatory factors could contribute to the baseline levels of HERG expression in MCF-7 cells. In comparison, estrogen and fludarabine, alone or in combination, did not alter the surface expression of HERG channel in the estrogen receptor negative MDA-MB-231 cell line (Fig. 3.4 B). The total HERG expression (middle blots of A and B) did not show significant alteration in the presence of estrogen or fludarabine or both treatments. These data indicate that a STAT1-dependent HERG upregulation exists in ER+ human breast cancer cells.

Figure 3.4

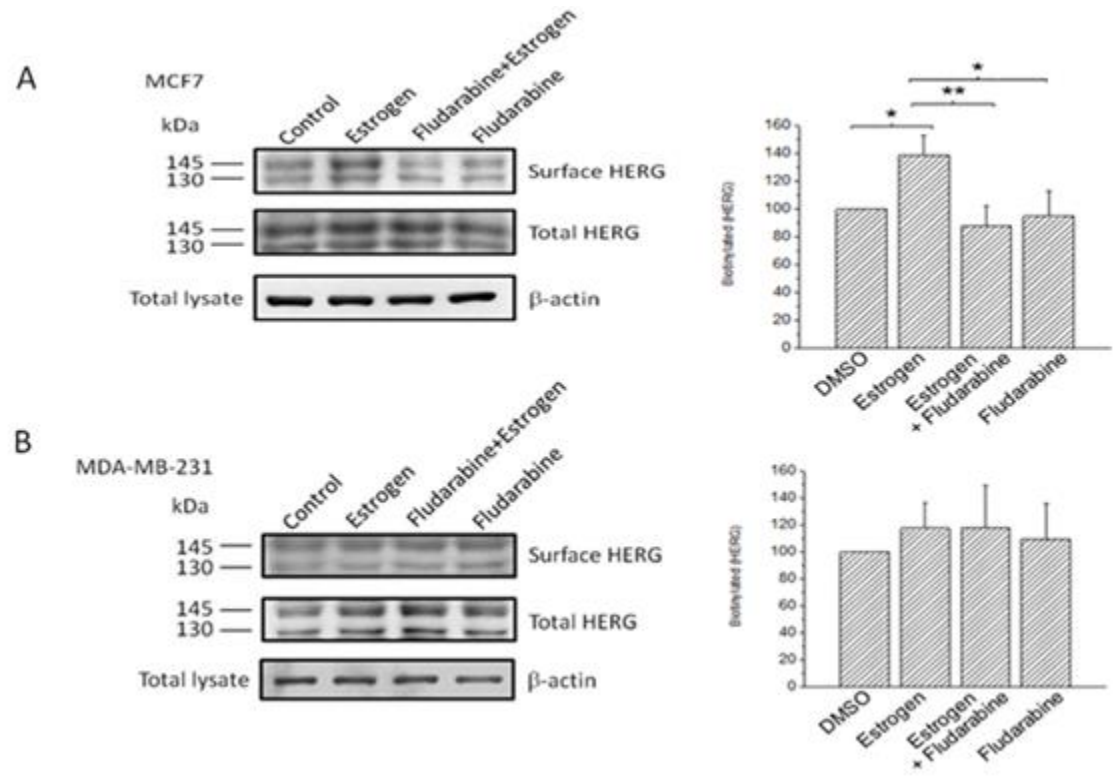


Figure 3.4 Surface upregulation of HERG potassium channels after estrogen treatment. A. Estrogen (3μM, 24h) significantly increased biotinylated HERG in MCF-7 human breast cancer cells. The selective STAT1 inhibitor fludarabine (50μM) prevented the HERG surface upregulation induced by estrogen. B. Estrogen did not significantly increase biotinylated HERG in MDA-MB-231 cells. The HERG bands in total whole cell lysates (middle of blots) did not differ significantly with the various treatment groups. HERG signals from total lysate (middle) were normalized by beta actin signals (bottom blots), and these normalized values were then used to normalize the biotinylated HERG signals. The values are means ± SEM and they are representative of 6 independent experiments. * $P<0.05$, ** $p<0.01$.

3.5 Inhibition of HERG channel, STAT1, *src* or estrogen receptor prevents estrogen induced proliferation of breast cancer cell lines

Since we found earlier that HERG, STAT1, and *src* appeared to form a physical complex in the various human cancer cell lines, we then performed a cell proliferation assay to assess the functional consequence of inhibiting HERG, STAT1, *src* or estrogen receptors. Here we found that estrogen (3μM, 48h) significantly increased cell proliferation in ER+ (MCF-7 and T47D) human breast cancer cell lines but not in ER- (MDA-MB-231 and BT-20 cells) (Fig. 3.5.1). Estrogen-induced cell proliferation was prevented in MCF-7 and T47D cells with pre-treatment of the cell lines with the selective HERG channel blocker, E4031 (10μM). Interestingly, E4031 alone also inhibited cell proliferation of MDA-MB-231 and BT-20 cell lines at 48h (Fig. 3.5.1). Together with the above biochemical and imaging results, these proliferation data indicate that HERG function can be upregulated by estrogen stimulation to promote proliferation of ER+ cancer cells, but that blocking HERG function alone is insufficient to reduce cell proliferation of ER- cancer cell lines.

Figure 3.5.1

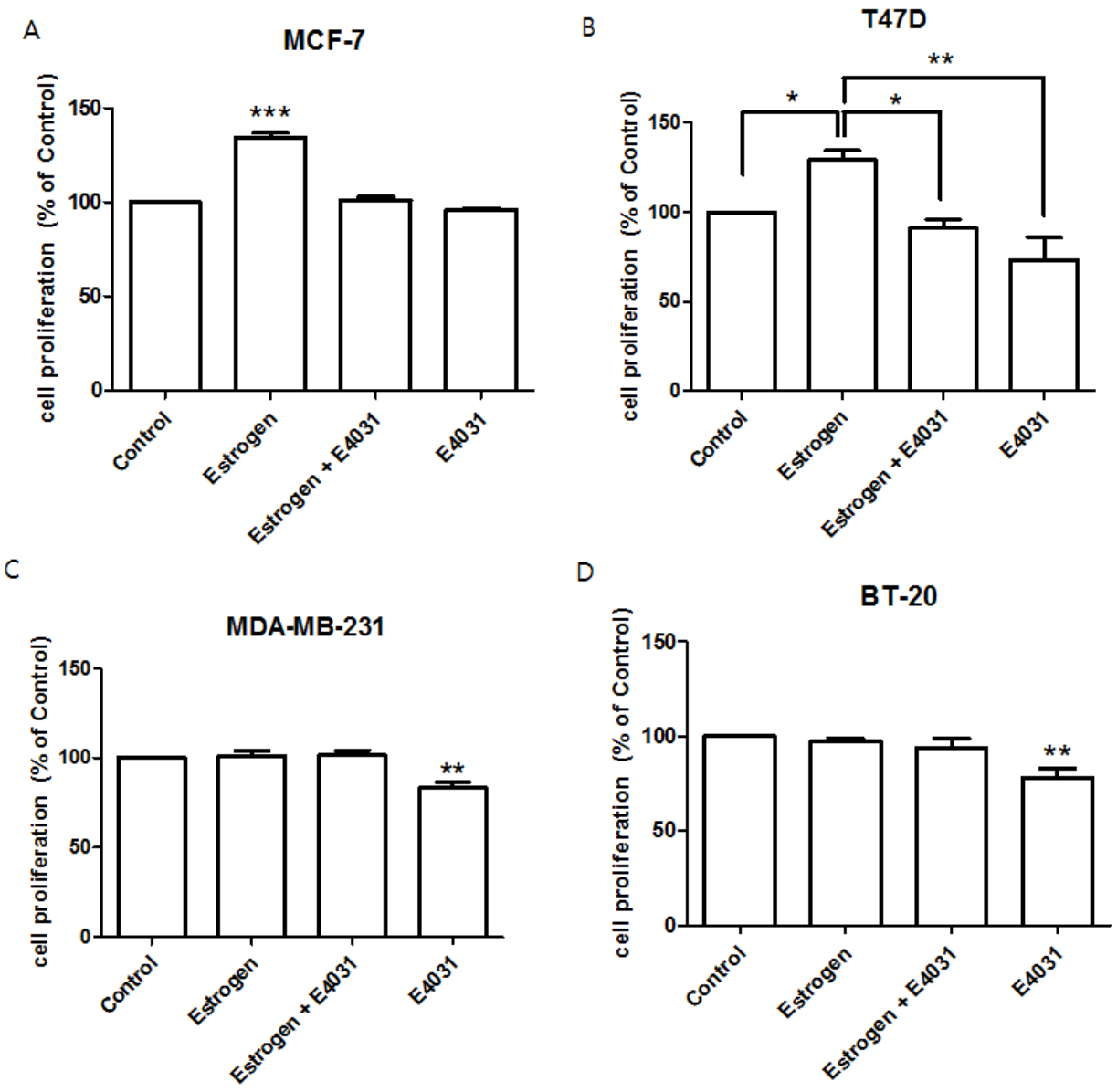


Figure 3.5.1 Cell proliferation assay and block of estrogen-induced proliferation by HERG channel blocker (E4031) in MCF-7, MDA-MB-231, BT-20 and T47D cells. Estrogen (3µM, 48h) significantly increased cell proliferation in MCF-7 and T47D (A and B) human breast cancer cells. The selective HERG channel inhibitor (E4031, 10µM) prevented the estrogen-induced cell proliferation 48h after estrogen stimulation of MCF-7 and T47D cells. Estrogen did not significantly increase cell proliferation in the ER- MDA-MB-231 and BT-20 cells (C and D). However, E4031 was able to prevent baseline proliferation of MDA-MB-231 and BT-20. One-way ANOVA followed by Student-Newman-Keuls post-hoc multiple comparison test was used

for statistical analyses of these proliferation assay data. Significances are indicated as follows, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Values are means ± standard deviations from 4 independent experiments.

Next, since STAT1 activation after estrogen receptor stimulation increased HERG surface expression, we hypothesized that inhibiting STAT1 function will result in inhibition of estrogen-induced cell proliferation of ER+ human breast cancer cell lines. Figure 3.5.2 shows that inhibition of STAT1 activation with fludarabine (50μM) significantly inhibited cell proliferation induced by estrogen after 48 hours in MCF-7 and T47D cells (Fig. 3.5.2 A and B). However, fludarabine alone also decreased baseline cell proliferation in all 4 breast cancer cell lines (Fig. 3.5.2 B) with T47D showing the greatest sensitivity to fludarabine's effect on cell proliferation.

Figure 3.5.2

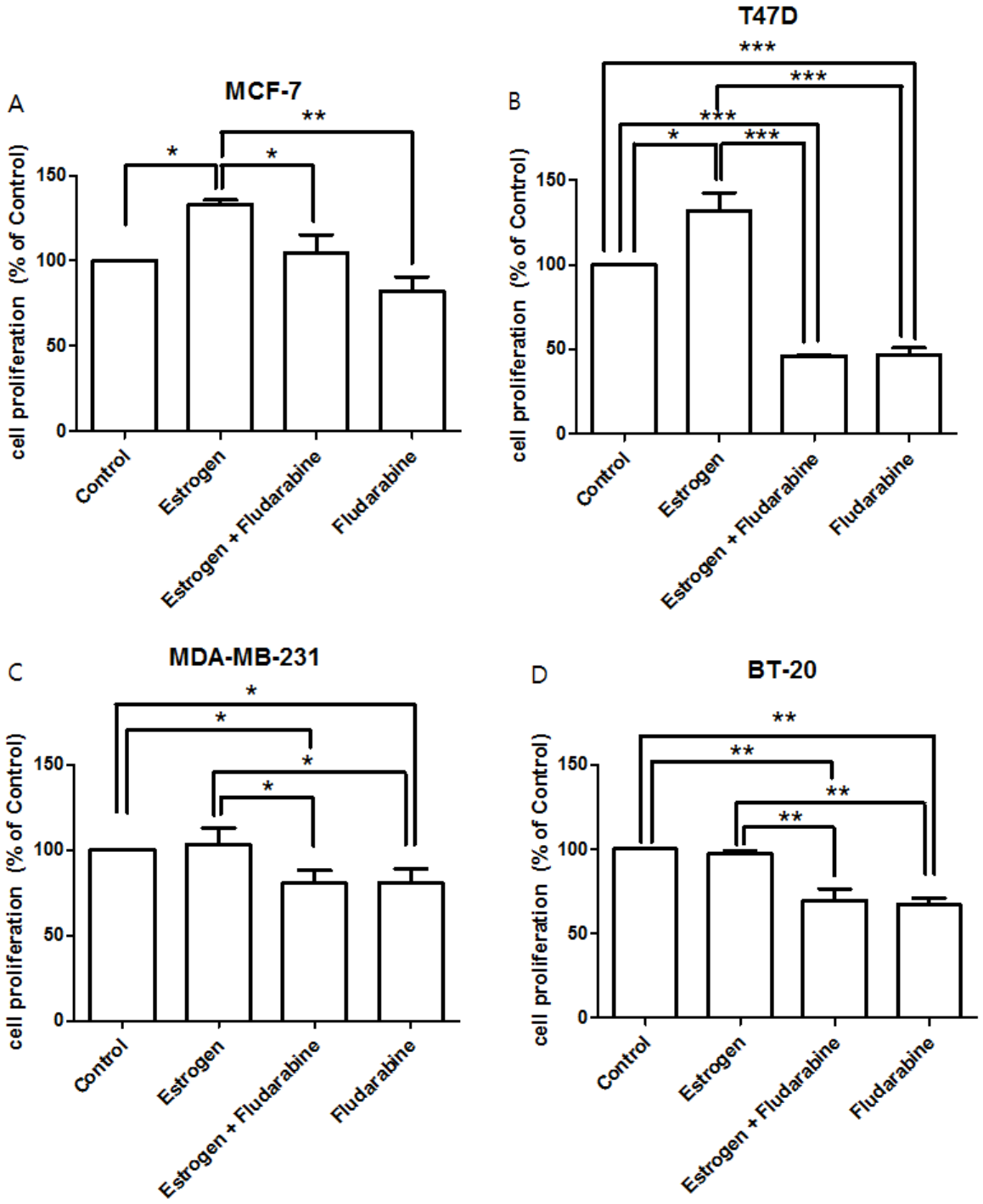

A
MCF-7
cell proliferation (% of Control)
150
100
50
0
*
*
**
Control
Estrogen
Estrogen + Fludarabine
Fludarabine
B
T47D
***
***
***
*
***
cell proliferation (% of Control)
150
100
50
0
Control
Estrogen
Estrogen + Fludarabine
Fludarabine
C
MDA-MB-231
*
*
*
*
cell proliferation (% of Control)
150
100
50
0
Control
Estrogen
Estrogen + Fludarabine
Fludarabine
D
BT-20
**
**
**
**
cell proliferation (% of Control)
150
100
50
0
Control
Estrogen
Estrogen + Fludarabine
Fludarabine

Figure 3.5.2 Cell proliferation assay and block of estrogen-induced proliferation by STAT1 inhibitor (fludarabine) in MCF-7, MDA-MB-231, BT-20 and T47D cells. Estrogen (3μM, 48h) significantly increased cell proliferation in MCF-7 and T47D (A and B) human breast cancer cells. Fludarabine (50μM) prevented the estrogen-induced cell proliferation 48h after estrogen stimulation of MCF-7 and T47D cells. Estrogen did not significantly increase cell proliferation in the estrogen receptor-negative MDA-MB-231 and BT-20 cells (C and D). However, fludarabine was able to prevent baseline proliferation of MDA-MB-231 and BT-20. One-way ANOVA followed by Student-Newman-Keuls post-hoc multiple comparison test was used for statistical analyses of these proliferation assay data. Significances are indicated as follows, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Values are means ± standard deviations from 4 independent experiments.

To determine whether the effects of estrogen on HERG-dependent cell proliferation was indeed sensitive to estrogen receptor antagonism, we tested whether inhibition of estrogen receptors with a non-specific estrogen receptor antagonist (ICI 182 780) also decreased estrogen-induced proliferation of MCF-7 and T47D cells. (Fig. 3.5.3 A and B). ICI alone or in combination with estrogen inhibited cell proliferation of MCF-7 and T47D, but did not affect cell proliferation of estrogen receptor negative cell lines (Fig. 3.5.3). Interestingly, ICI alone inhibited baseline proliferation of T47D, similar to the effect of E4031 and fludarabine on this cell line.

Figure 3.5.3

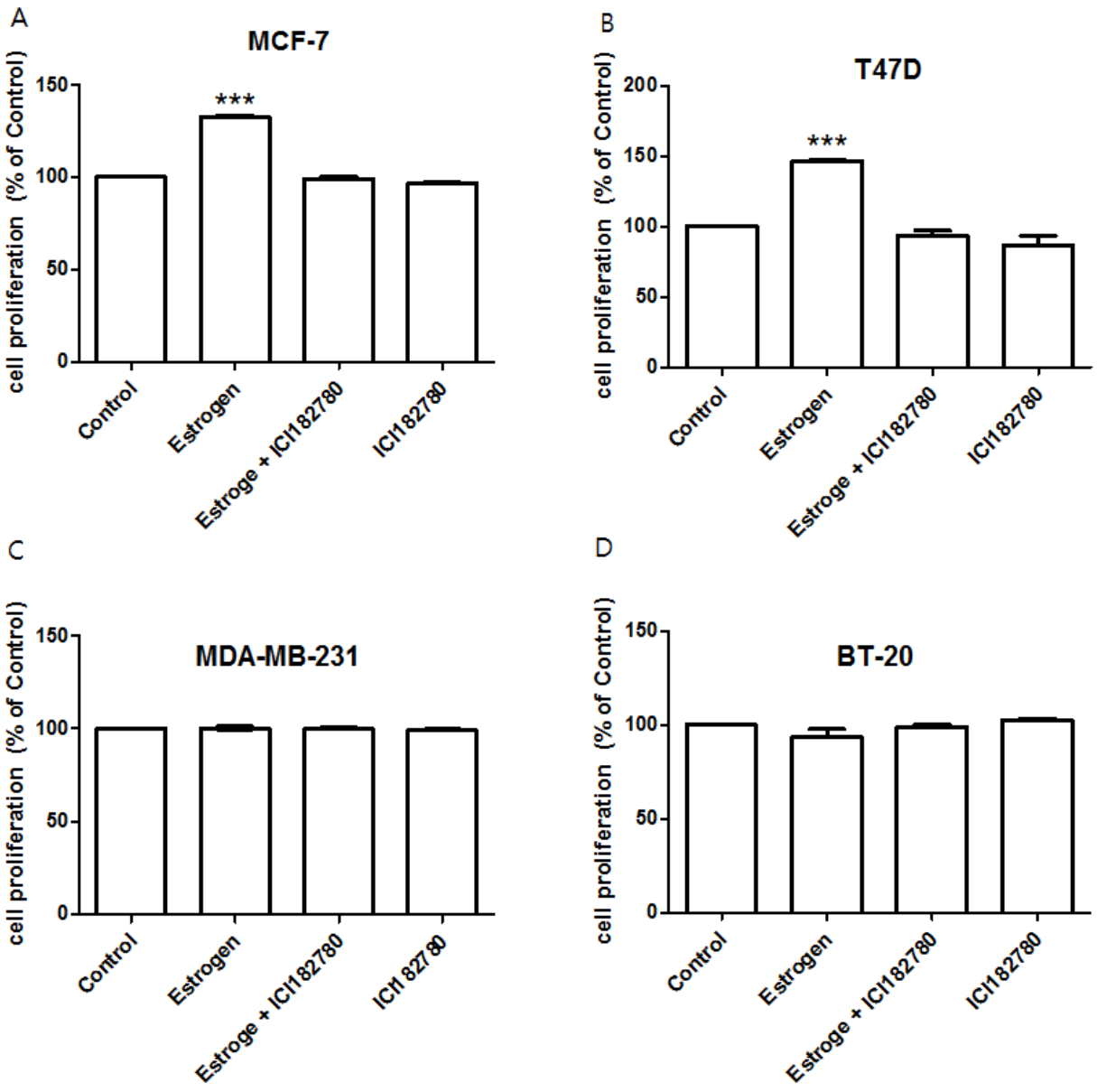


Figure 3.5.3 Cell proliferation assay and block of estrogen-induced proliferation by estrogen receptor blocker (ICI 182 780) in MCF-7, MDA-MB-231, BT-20 and T47D cells. Estrogen (3μM, 48h) significantly increased cell proliferation in MCF-7 and T47D (A and B) human breast cancer cells. ICI 182 780 (5μM) prevented the estrogen-induced cell proliferation 48h after estrogen stimulation of MCF-7 and T47D cells. Estrogen did not significantly increase cell proliferation in the ER- MDA-MB-231 and BT-20 cells (C and D). One-way ANOVA followed

by Student-Newman-Keuls post-hoc multiple comparison test was used for statistical analyses of these proliferation assay data. Significances are indicated as follows, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Values are means ± standard deviations from 4 independent experiments.

Previously, HERG function was shown to be upregulated by endogenous *src* activation [77], and we have shown above that HERG interacts with *src*. Therefore, we tested the hypothesis that HERG-dependent cell proliferation can be inhibited by *src* inhibitors. As shown in Figure 3.5.4, *src* inhibitors herbimycin A and PD 166 285 both significantly reduced estrogen-induced cell proliferation.

Figure 3.5.4

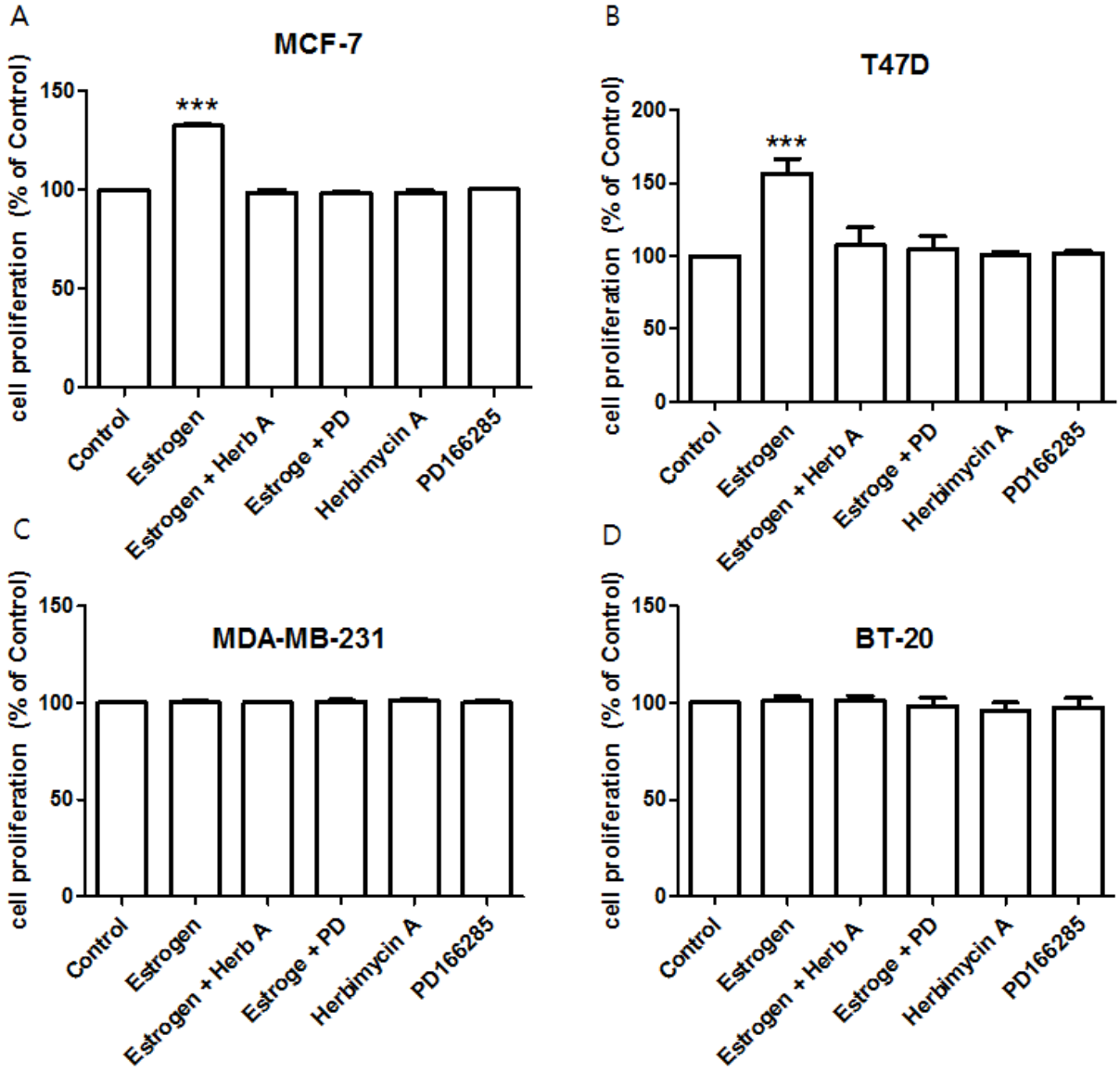


Figure 3.5.4 Cell proliferation assay and block of estrogen-induced proliferation by *src* inhibitors (herbimycin A and PD 166 285) in MCF-7, MDA-MB-231, BT-20 and T47D cells. Estrogen (3µM, 48h) significantly increased cell proliferation in MCF-7 and T47D (A and B) human breast cancer cells. Herbimycin A (3µM) and PD 166 285 (100nM) prevented the estrogen-induced cell proliferation 48h after estrogen stimulation of MCF-7 and T47D cells. Estrogen did not significantly increase cell proliferation in the estrogen receptor-negative MDA-MB-231 and BT-20 cells (C and D). One-way ANOVA followed by Student-Newman-Keuls post-hoc multiple comparison test was used for statistical analyses of these proliferation assay data. Significances are indicated as follows, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Values are means ± standard deviations from 4 independent experiments.

3.6 Probing HERG-STAT1 interaction with FR-peptide

3.6.1 Cellular Uptake of FR-peptide

To determine whether the SH2 domain of STAT1 is required for HERG-STAT1 binding, we developed a peptide mimetic of the STAT1 SH2 domain. This 28 amino acid peptide corresponds to the SH2 domain of STAT1 and has a phenylalanine at the beginning and an arginine at the end (hence, this peptide is called FR-peptide). First, we determined whether the FR-peptide was cell permeable by incubating MCF-7 cells with the labelled or non-labelled peptide for 48h before performing immunocytochemistry. We fluorescently tagged the N terminus of FR-peptide using 5-carboxyfluorescein. Confocal microscopy of MCF-7 cells showed a significant intracellular accumulation of green fluorescence in cells treated with 5-carboxyfluorescein-labelled FR-peptide (Fig. 3.6), indicating that the labelled peptide had been successfully incorporated by the cells. The green fluorescence showed a distinct punctate pattern that was reminiscent of the HERG or STAT1 punctate staining. In contrast, cells treated with the non-labelled FR-peptide or just 5-carboxyfluorescein displayed little or no intracellular presence of green fluorescence. Moreover, the labelled peptide was localized near the plasma membrane and in intracellular compartments with the punctate pattern characteristic of HERG or STAT1 labelling pattern. Furthermore, the labelled scrambled FR-peptide also appeared to be accumulated but the staining pattern appears to be homogeneous, consistent with the lack of specific intracellular binding of this peptide. These results indicate that the FR-peptide crosses the plasma membrane (and to a lesser extent, the nuclear envelop) and may interact with the endogenous binding partners, presumably including the STAT1 SH2 domain region (i.e., STAT1 SH2 domain dimerizing with the FR-peptide) or the phosphotyrosine-enriched region(s) of the HERG channels.

Figure 3.6

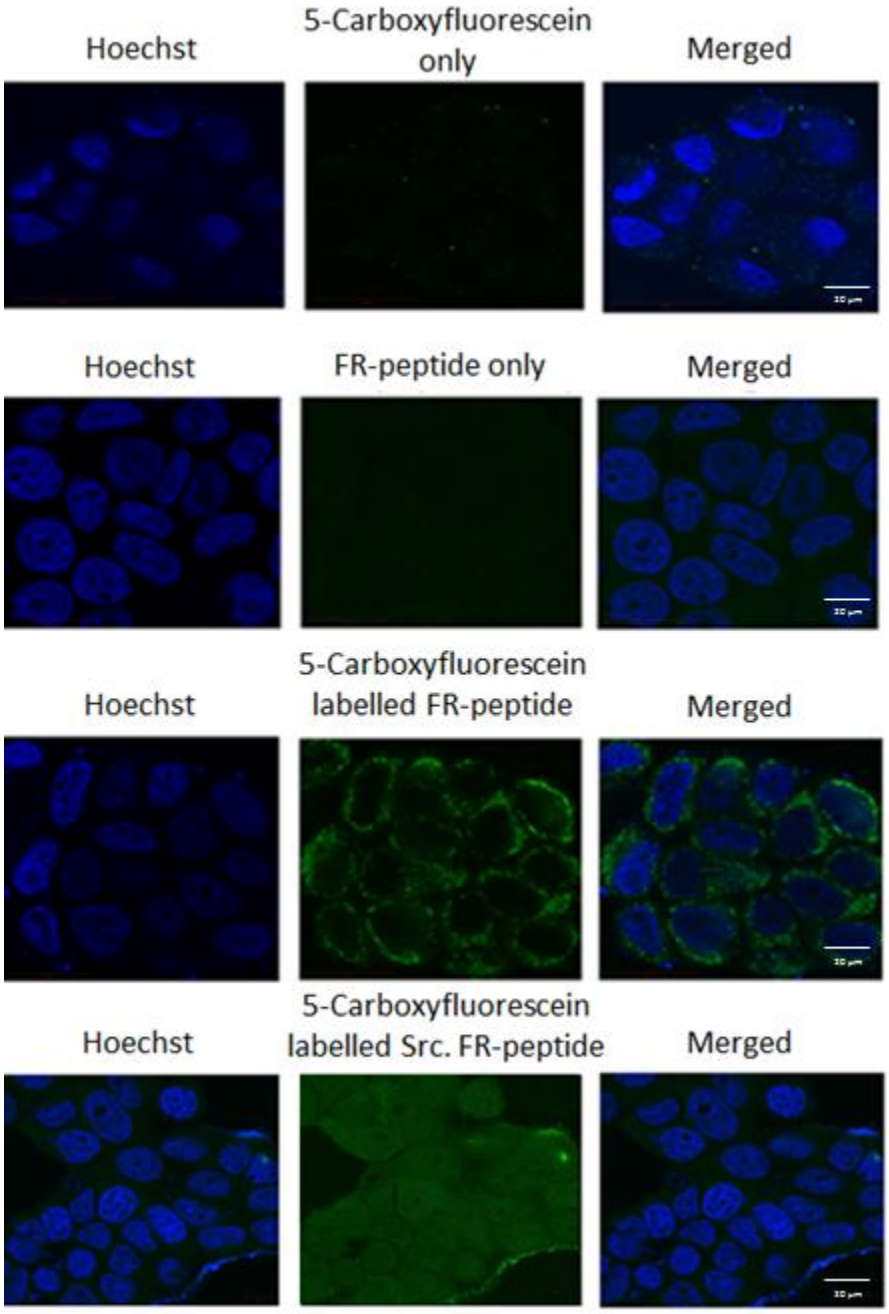


Figure 3.6 Cellular uptake of FR-peptide by MCF-7 cells. Fluorescence imaging of MCF-7 cells following treatment with 30 μM of 5-carboxyfluorescein labelled FR-peptide for 48 hours (third row) shows significant intracellular punctate green fluorescence. Incubations with either non-labelled FR-peptide (second row) or 5-carboxyfluorescein only (top row) did not show any fluorescence labelling. A 5-carboxyfluorescein labelled scrambled FR-peptide (bottom row) showed homogeneous intracellular accumulation of green fluorescence.

3.7 Effects of FR-peptide on HERG-STAT1 interaction in MCF-7 cells

Co-immunoprecipitation studies showed an increase in HERG and STAT1 interaction 48h after estrogen treatments (3μM) of MCF-7 cells (see above). We predicted that FR-peptide pre-incubation to disrupt the potential HERG-STAT1 binding prior to estrogen treatments should dramatically reduce the HERG-STAT1 interaction. Consistent with this suggestion, we showed that the FR-peptide (30μM) alone or in combination with estrogen stimulation (48h) significantly decreased the level of HERG and STAT1 interaction as suggested by the forward and reverse co-immunoprecipitation studies (Fig. 3.7A,B). Moreover, even with only 4h preincubation with the FR-peptide alone or in combination with estrogen treatment, we observed decreased or complete prevention of HERG-STAT1 binding (see last two lanes in the reverse co-immunoprecipitations, Fig 3.7D). The decreased co-IP observed at 48h for estrogen + FR-peptide treatment group could be explained by altered expression of either HERG or STAT1. Therefore, we performed Western blotting of MCF-7 lysates using HERG antibody (middle blots). However, there were no significant differences in the total expression of HERG in presence of estrogen with or without FR-peptide (middle blots) when normalized to GAPDH (quantification not shown). To determine whether the decreased HERG-STAT1 interaction involves disruption of STAT1 SH2 domain binding to HERG, we tested whether the FR-peptide shows similar effects after only 4h (when it is expected that significant change in protein translation is unlikely, or no significant protein degradation has taken place). As shown in Fig. 3.7 C, D, estrogen did not significantly increase HERG levels or HERG-STAT1 interaction. However, FR-peptide alone appeared to disrupt endogenous HERG-STAT1 interaction after 4h peptide incubation (Fig 3.7D), when HERG levels appear to be unaltered. However, the reverse co-IP (Fig. 3.7C) showed no decrease in HERG and STAT1 in presence of FR-peptide alone. Together, FR-peptide may affect HERG-STAT1 interaction as early as 4 hours after initial incubation with the peptide.

Figure 3.7

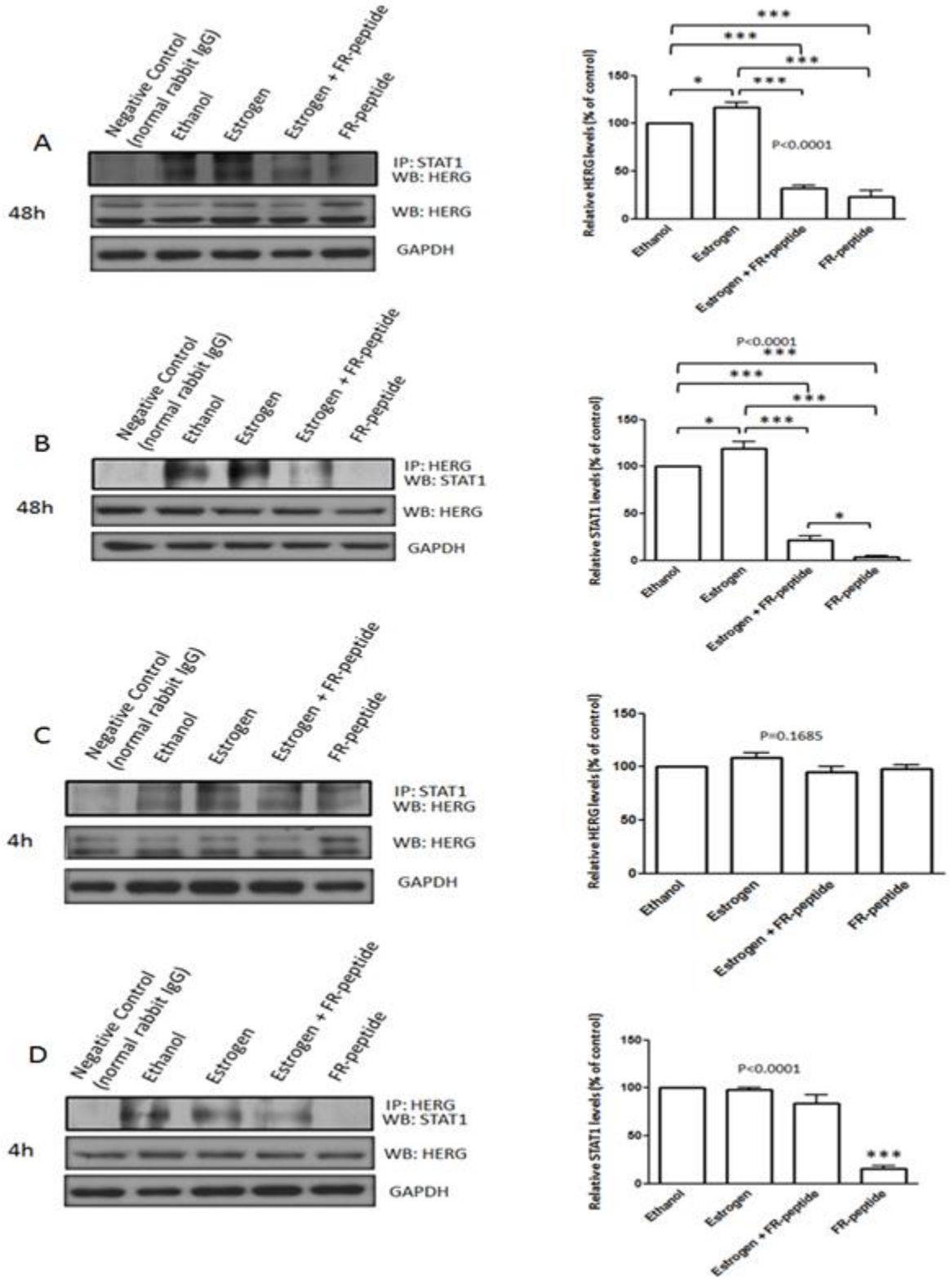

A
Negative Control (normal rabbit IgG)
Ethanol
Estrogen
Estrogen + FR-peptide
FR-peptide
IP: STAT1
WB: HERG
48h
WB: HERG
GAPDH
Relative HERG levels (% of control)
P<0.0001
Ethanol
Estrogen
Estrogen + FR+peptide
FR-peptide
B
IP: HERG
WB: STAT1
48h
WB: HERG
GAPDH
Relative STAT1 levels (% of control)
P<0.0001
Ethanol
Estrogen
Estrogen + FR-peptide
FR-peptide
C
IP: STAT1
WB: HERG
4h
WB: HERG
GAPDH
Relative HERG levels (% of control)
P=0.1685
Ethanol
Estrogen
Estrogen + FR-peptide
FR-peptide
D
IP: HERG
WB: STAT1
4h
WB: HERG
GAPDH
Relative STAT1 levels (% of control)
P<0.0001
Ethanol
Estrogen
Estrogen + FR-peptide
FR-peptide

Figure 3.7 FR-peptide prevented the estrogen-induced HERG and STAT1 interaction in estrogen receptor-positive MCF-7 human breast cancer cells. A. STAT1 immunoprecipitates showed HERG doublets near 130 and 150 kDa at 48h of treatments. Estrogen (3μM, 48h) significantly increased interaction of HERG and STAT1 (lane 2). FR-peptide (30μM, 48h) significantly reduced HERG and STAT1 interactions at 48h. B. the reverse co-immunoprecipitation showed that HERG immunoprecipitates contained STAT1 proteins near the predicted 87 kDa molecular mass at 48h of treatments. Estrogen increased interaction of HERG and STAT1 (lane 3), and FR-peptide (30μM, 48h) reduced HERG and STAT1 interactions. C, D. co-immunoprecipitation studies of STAT1 (C) or HERG (D) after 4h treatment of estrogen with or without FR-peptide showed that a similar inhibition of co-precipitation between HERG and STAT1 was observed after 4h peptide incubation. In D, the HERG immunoprecipitate did not appear to contain STAT1 after 4h peptide incubation (last lane).

3.8 Effects of FR-peptide on regulation of surface HERG channels in MCF-7 cells

Having shown earlier that the STAT1 inhibitor fludarabine decreased the estrogen-induced HERG surface upregulation, we predicted that the FR-peptide would produce a similar effect by inhibiting HERG surface expression. As shown in Figure 3.8 A, Western blot analyses of surface biotinylated proteins confirmed that the FR-peptide was indeed effective in preventing HERG surface upregulation after 48h estrogen treatment (3 μM) of MCF-7 cells (n=4). In contrast, the 4h estrogen treatments with or without the FR-peptide did not significantly alter the level of HERG surface expression, likely owing to the very short time period to allow for any appreciable level of newly translated HERG protein to be observed.

Figure 3.8

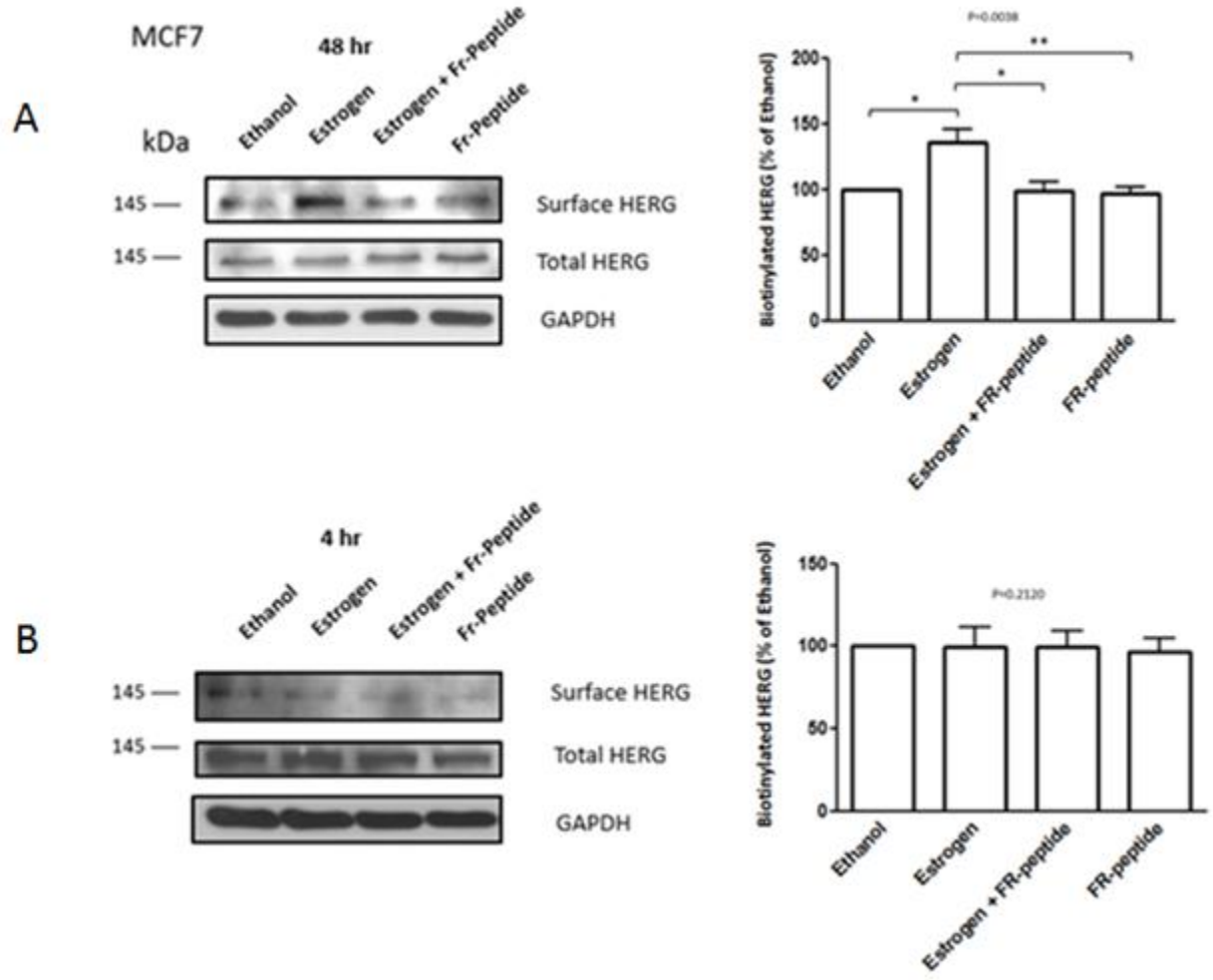


Figure 3.8 Surface upregulation of HERG potassium channels 48 hours after estrogen treatment in estrogen receptor-positive MCF-7 human breast cancer cells. A. Estrogen (3μM, 48h) significantly increased surface biotinylated HERG in MCF-7 cells. FR-peptide (30μM) prevented the HERG surface upregulation by estrogen. B. Estrogen did not significantly increase biotinylated HERG in MCF-7 cells after 4 hours. The HERG bands in total whole cell lysates did not differ significantly with the various treatment groups. The values are means ± SEM and they are representative of 4 independent experiments.

3.9 Effects of FR-peptide on proliferation of MCF-7 cells

Earlier we showed that estrogen (3µM, 48h) significantly increased cell proliferation and fludarabine prevented this effect in ER+ MCF-7 human breast cancer cells. However, estrogen did not affect cell proliferation in the ER- MDA-MB-231 cell line. Having shown earlier that FR-peptide decreased HERG surface expression, we hypothesized that the FR-peptide would prevent binding of endogenous STAT1 to HERG channels, and therefore we predicted that that estrogen-induced proliferation would also be decreased by FR-peptide pre-treatments. In MCF-7 cells, FR-peptide indeed prevented the estrogen-induced proliferation at all the tested concentrations (10µM, 30µM and 100µM) (n=11) as shown in Figure 3.9.1. However, the peptide did not significantly decrease the proliferation in MDA-MB-231 cells (n=9), despite the demonstration of a positive co-precipitation of HERG and STAT1 in this cell line (Figure 3.2 A and B). Scrambled version of FR-peptide was synthesized and used in proliferation assay experiments as a negative control for FR-peptide (Labelled scrambled peptide was also shown to be cell permeable). In contrast to FR-peptide, the scrambled FR-peptide did not have any effect on proliferation of MCF-7 or MDA-MB-231 cell lines (Fig. 3.9.2).

Figure 3.9.1

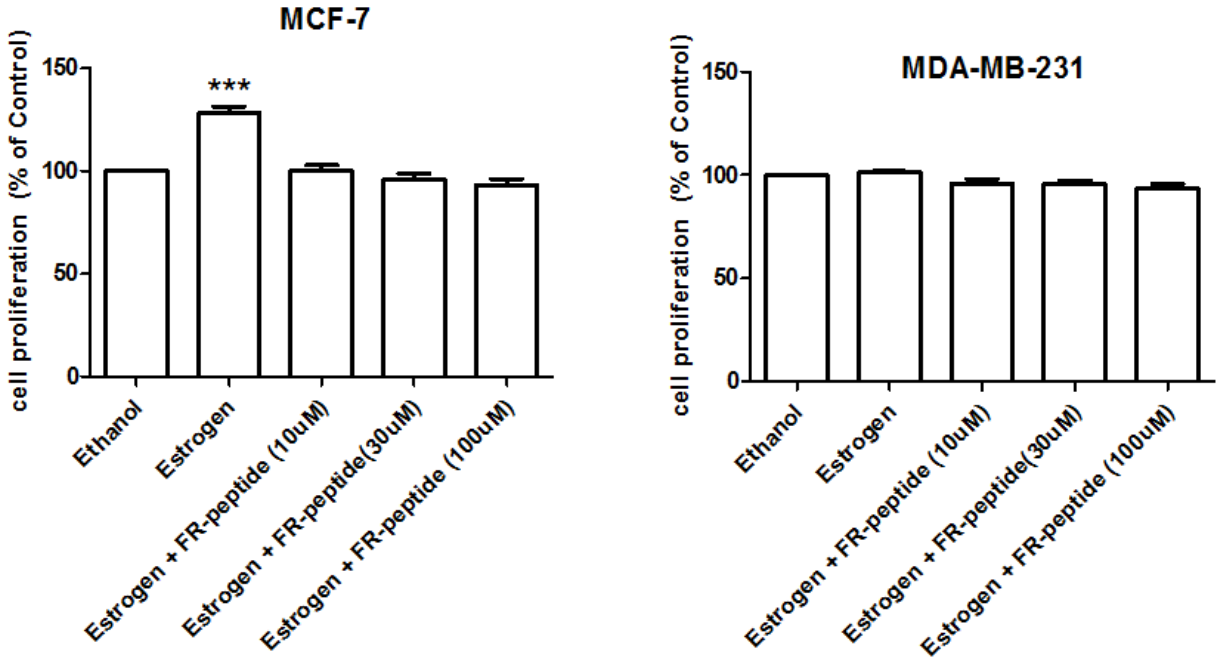


Figure 3.9.1. FR-peptide prevented estrogen-induced proliferation of MCF-7 breast cancer cell line. Estrogen (3μM, 48h) significantly increased cell proliferation in MCF-7 human breast cancer cells. Three different concentrations of FR-peptide (10, 30 and 100μM) prevented the estrogen-induced cell proliferation 48 h after estrogen stimulation. Estrogen did not significantly increase cell proliferation in the estrogen receptor-negative MDA-MB-231 cells. One-way ANOVA followed by Student-Newman-Keuls method was used in statistical analyses of these proliferation assay data. Significances are indicated as follows, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Values are means ± standard deviations from 4 independent experiments.

Figure 3.9.2

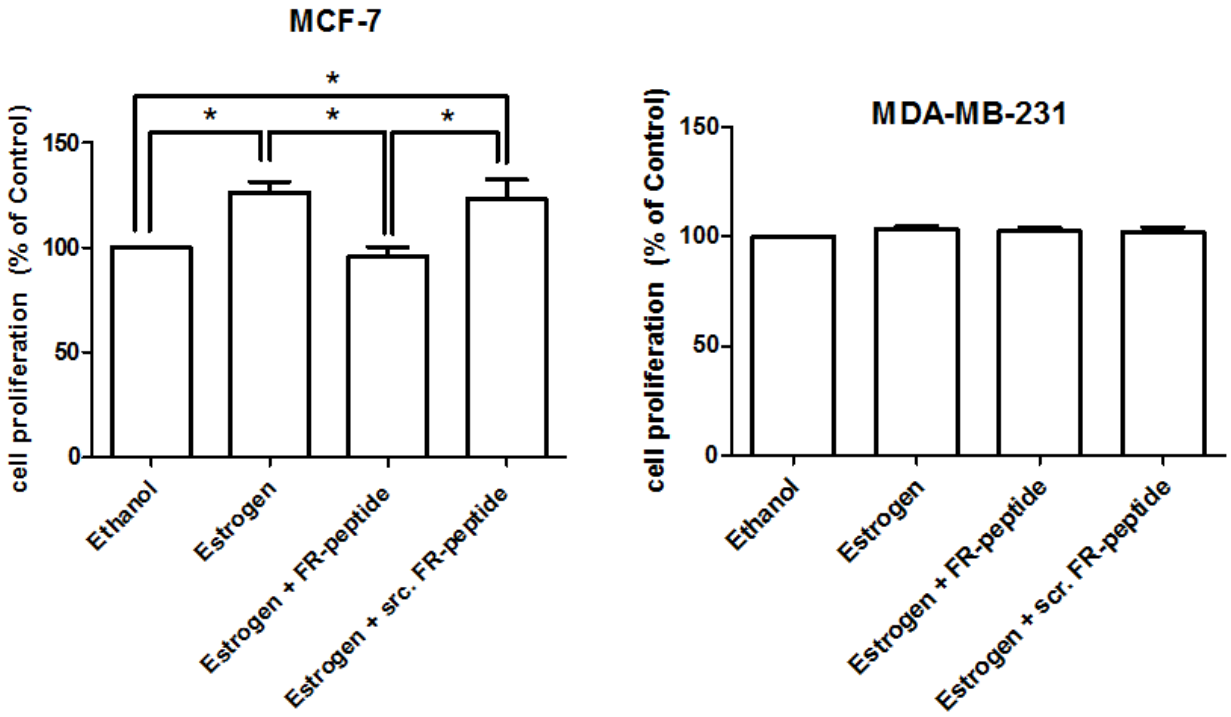


Figure 3.9.2. FR-peptide, but not the scrambled version of this peptide, prevented estrogen-induced proliferation of MCF-7 breast cancer cell line. FR-peptide (30µM) prevented the estrogen-induced cell proliferation at 48h incubation but the scrambled FR-peptide did not prevent the estrogen-induced proliferation in MCF-7 cells. One-way ANOVA followed by Student-Newman-Keuls post-hoc multiple comparisons test was used in statistical analyses of these proliferation assay data. Significances are indicated as follows, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Values are means ± standard deviations from 3 independent experiments.

3.10 STAT1 and HERG do not interact in normal cardiac or brain tissue

HERG which encodes the α-subunit of the rapid component of the delayed rectifier K^+ current (I_{Kr}), is known to play a crucial role in regulating the repolarization of the cardiac action potential [58-60]. Therefore, any disturbance in its conductance or expression level prolongs QT intervals and increases the risk of lethal arrhythmia. Thus, to determine whether the estrogen-induced and STAT1-dependent upregulation of HERG channels is important in cardiac tissue, we then examined whether the interaction between STAT1 and HERG exists in cardiac tissue and other normal tissue from rat. STAT1 is ubiquitously expressed, including neuronal and cardiac cells, and is known to regulate the immune system to prevent pathogen infections [102, 132]. Since STAT1 is only upregulated upon adaptive immune responses to infection [133], we predicted that STAT1 and HERG expression in healthy cardiac or brain tissue would be comparatively lower compared to cancerous tissue, and this will be expected to result in little or no interaction between HERG and STAT1 in healthy tissue. As we predicted, we did not observe STAT1 and HERG co-immunoprecipitation in normal cardiac or brain tissue (Fig. 3.10), but we observed co-precipitation in MCF-7 lysates as shown earlier (Fig. 3.2 and 3.4). We also did not observe the effect of 3μM estrogen applied for 24h on increased HERG expression or HERG-STAT1 interaction in isolated rat cardiomyocyte cultures (data not shown). Immunoprecipitation with the STAT1 antibody (A, lanes 2-4) did not co-precipitate HERG proteins in rat ventricular lysate (lane 2) or rat brain lysate (lane 3). However, MCF-7 cell lysate showed a positive co-precipitation between and HERG and STAT1. Reverse co-immunoprecipitation studies (B) also showed that HERG immunoprecipitates contained STAT1 (lane 4) from MCF-7 lysate, but rat ventricular lysate (lane 2) or rat brain lysate (lane 3) did not reveal co-precipitation of HERG and STAT1. Taken together, these data indicate that HERG-STAT1 interaction may be specific to cancerous tissue.

Figure 3.10

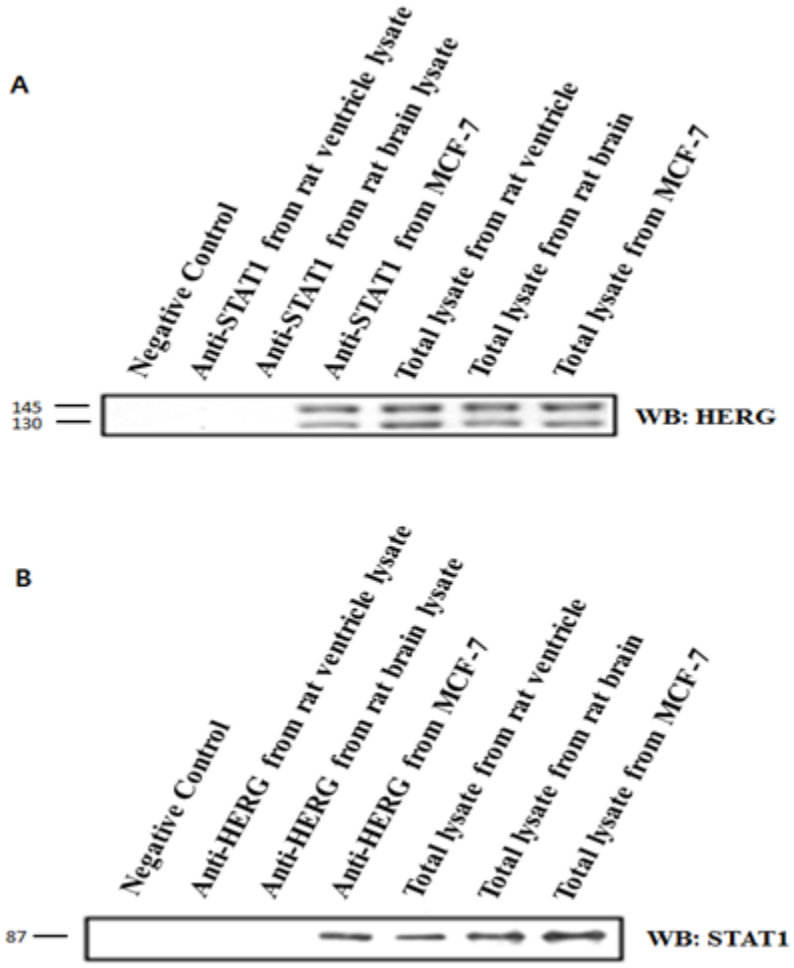


Figure 3.10 Lack of co-immunoprecipitation of HERG and STAT1 in normal rat cardiac and brain tissue. A. STAT1 immunoprecipitates from MCF-7 cell lysate (lane 4) and positive control lysates from rat ventricle, rat brain, and MCF-7 (lanes 5-7) showed HERG doublets at near 130 and 150 kD. However, lysates from normal rat ventricle and brain did not show an interaction between HERG and STAT1 (lanes 2 and 3 vs. lane 4). B. HERG immunoprecipitates from MCF-7 cell lysate (lane 4) and positive control lysates (lane 5-7) showed STAT1 band near 87 kDa. This reverse co-immunoprecipitation study also did not show an interaction between HERG and STAT1 in normal rat tissue (lanes 2 and 3 vs. lane 4). Negative controls (lane 1) using MCF-7 lysates represent the omission of the immunoprecipitating antibody, and rabbit IgGs were used instead for all blots.

4. DISCUSSION

The pathways involved in estrogen-stimulated tumour progression vary widely and there is still a scarcity of knowledge on the molecular targets in estrogen-dependent breast cancer. Even though it is known that HERG channel is overexpressed in various breast cancer cell lines [90, 130], little is known about the mechanism of HERG overexpression in breast cancer.

Here, we propose a novel downstream signalling pathway of estrogen involving STAT1 and HERG interactions in ER positive breast cancers. The data reported here show that HERG and STAT1 form a multiprotein complex in breast cancer that can be increased by estrogen stimulation. Moreover, our results showed that the HERG-STAT1 interaction is important for surface expression of HERG. This new knowledge is expected to contribute to the development of novel therapeutic targets for ER+ breast cancers, by minimizing the potential side effects of blocking HERG channels expressed in cardiac tissue.

The expression of HERG has been previously studied in various tumour cell lines in order to examine the underlying mechanism of HERG regulation of cancer cell proliferation. Regulation of HERG current could result from any of the mechanisms listed: (i) change in number of channels expressed on the cell surface, (ii) change in single-channel conductance or (iii) altered kinetics of the channels. In cancer cells where the expression level of HERG channel is significantly higher compared to their normal counterparts, the first mechanism can account for increase in HERG function. The data reported here also demonstrate that HERG is overexpressed in cancerous breast tissue compared to the normal breast tissue. Furthermore, expression of HERG was confirmed in various breast cancer cell lines with differential estrogen receptor expression. However, our studies do not rule out that whether estrogen stimulation could alter the biophysical properties of HERG that promote increased HERG channel conductances. Future electrophysiological studies will be

required to test this possibility, especially to examine whether STAT1 binding to HERG at the plasma membrane is sufficient to alter the gating kinetics of HERG channels.

In breast cancer, the expression level of ERα is increased and aberrant estrogen signalling is often observed [18-20]. We hypothesized that estrogen signalling may be involved in HERG expression since non-genomic activation of ERs has a downstream target, namely the *src* tyrosine kinase which is known to regulate HERG current [77]. It has been previously reported that HERG current is positively regulated by *src* [77], whereas it is negatively regulated by the tyrosine phosphatase SHP-1 [125]. This finding demonstrated the importance of tyrosine phosphorylation of HERG on its function. Furthermore, HERG tyrosine phosphorylation may provide an explanation for the up-regulation of HERG channels in breast cancer where *src* is constitutively active [134, 135]. In pilot studies, we showed that *src* inhibitors or the FR-peptide decreases HERG phospho-tyrosine levels as well as HERG surface expression. Our present study showed that estrogen-induced cell proliferation was decreased by *src* inhibitors, fludarabine and FR-peptide. Our results showing that *src* inhibitors are effective in preventing estrogen-induced cell proliferation are in line with our proposed mechanism that estrogen contributes to increased *src*-mediated tyrosine phosphorylation and function of HERG channels. Since we also observed increased expression of HERG upon estrogen stimulation, we suggest that both increased HERG function (by increased tyrosine phosphorylation by *src* and potentially by increased protein-protein interaction with STAT1) and increased HERG surface expression both contribute to the estrogen-induced cell proliferation in ER+ human breast cancer cell lines.

Moreover, s*rc* family kinases activate STAT-family proteins which are also known to regulate proliferation of cancer cells [126-128]. Therefore, aberrant estrogen signalling in breast cancer could involve activation of STAT1 through *src*. Many studies have demonstrated that not

only activation but also expression level of STAT1 has an impact on tumour progression [117, 118, 124, 131]. The current knowledge on the expression level and function of STAT1 in breast cancer is controversial. Many studies have reported low levels of STAT1 found in ERα positive breast cancer [117, 118]. However, recent studies on STAT1 showed increased STAT1 expression and activity in breast cancer [124, 131]. In this study, our results showed that STAT1 is overexpressed in cancerous breast tissue compared to normal breast tissue. Furthermore, we showed that estrogen can induce an increase in the HERG-STAT1 interaction. Although we used ICI 182 780 to inhibit estrogen receptors and this was shown to be effective in reducing estrogen-induced HERG surface expression and cancer cell proliferation, it remains to be established which estrogen receptor subtype (ER alpha or beta) is responsible for the upregulation of STAT1 and HERG in ER+ human breast cancer cells. Future studies with ER subtype-specific antagonists or siRNA knockdown of either ER alpha or ER beta will aid in the elucidation of this signalling pathway that contributes to HERG-STAT1 interaction and proliferation in breast cancer cells.

To examine the effect of disrupting the HERG-STAT1 interaction, we used a peptide which encompasses the full 28 amino acid region of STAT1 SH2 domain and examined its potential therapeutic value in minimizing estrogen-induced HERG surface upregulation and, consequently, increased cell proliferation. Our results showed that FR-peptide was cell permeable and, indeed, was able to disrupt the HERG and STAT1 interaction in ER+ breast cancer cell line. Moreover, we observed that FR-peptide was able to prevent estrogen-induced upregulation of surface HERG channels. Our co-precipitation results also provided supporting evidence that HERG-STAT1 interaction occurs, in part, through STAT1 SH2 domain binding to phospho-tyrosine HERG residues, although the available data could not rule out that indirect interaction through an intermediary signalling protein may also account for the HERG-STAT1

binding. In previous studies [77, 125], there were a number of tyrosine residues identified that could serve as potential binding sites for the *src* SH2 domain. These regions include N-terminal tyrosine residues (Y54SRA, Y329RTI, and/or Y405SPF), S4-S5 linker Y544SEY, and several C-terminal tyrosine residues (Y814ARP, Y829CDL, Y847PEF, Y1013QEL, or Y1082SAV). Whether any or all of these tyrosine residues can be phosphorylated by endogenous tyrosine kinases remains to be established. It would also be important to establish whether any of these phospho-tyrosine residues could similarly serve as docking sites for multiple signalling proteins that have SH2 domains, including STAT-family of proteins, *src*, and others. Since we showed that HERG also co-precipitates with *src*, it will be important to test in future studies whether the *src* SH2 domain peptide could similarly prevent STAT1-HERG interactions. If so, this would indicate that estrogen signalling leads to *src* activation, which is presumably upstream of the HERG and STAT1 interaction. This might explain why the FR-peptide completely prevented the estrogen-induced cell proliferation but did not cause further reduction in cell proliferation below baseline (ethanol control in Fig. 3.9.1 and Fig. 2.9.2), as other potential HERG interacting proteins, such as *src*, continue to be activated and unaffected by the FR-peptide. Moreover, determination of the binding specificity of SH2 domains may not only extend to other signaling proteins (e.g., SH2 domains of Grb2, Cbl, or other *src*-family members, etc.), but also whether the SH2 domain of other STAT-family members mediate direct binding to the HERG phospho-tyrosine residues, will require further investigation.

It is likely that the disruption of HERG and STAT1 interaction by FR-peptide is in a form of competitive inhibition. FR-peptide, acting as a competitive inhibitor, may lower the number of available HERG proteins for STAT1 binding. Since HERG and STAT1 interaction appears to be important in inducing HERG overexpression, the ability to disrupt HERG and STAT1 interaction

raises the possibility that FR-peptide or a small molecule with similar effects can be used as a potential anti-cancer therapeutic.

There are several advantages of using FR-peptide over other HERG blockers as an intervention to prevent aberrant cell proliferation of ER positive breast cancer cells. One advantage of using FR-peptide is that this peptide may not produce any cardio-toxic side effects produced by direct blockade of cardiac-expressed HERG channels. Rather than directly blocking the HERG channels, our results showed that this peptide may allow the downregulation of HERG surface expression in ER+ breast cancers only. Also, the HERG-STAT1 interaction appears to occur exclusively in human breast cancers although it remains to be established whether the HERG-STAT1 interaction also occurs in other types of cancers. As shown previously, our results suggest that there are no native HERG-STAT1 interactions in normal brain or cardiac tissue based on our co-immunoprecipitation studies. Thus, the FR-peptide or a small molecule compound that mimics FR-peptide's effect should not be expected to have any deleterious effects on cardiac HERG regulation, thus avoiding the unwanted cardio-toxic side-effects associated with most drugs that fail the Food and Drug Administration (FDA) approval.

However, there needs to be more studies done on the use of FR-peptide. STAT3 is another member of the STAT-family proteins, which is also known to regulate proliferation of cancer cells [136-139]. STAT3 heterodimerizes with STAT1 in different cell types to exert its transcriptional effect in the nucleus [140, 141]. The use of FR-peptide may reduce the interaction between STAT1 and STAT3. Indeed, peptide mimetic of the SH2 domain of STAT3 has been shown to decrease the STAT1 and STAT3 dimerization in hematopoietic cancer cells [142]. Reducing the availability of STAT1 for STAT3-STAT1 dimerization may promote greater STAT3-STAT3 interaction which is known to have an apoptotic effect. Therefore, it is crucial to

study other potential side effects of using FR-peptide, specifically to determine whether these effects promote cell death in cancer cells or promote toxicity in other tissue. Furthermore, more analysis needs to be done on this peptide regarding its half-life and IC50 for more effective use of the peptide in future *in vivo* studies.

For the first time, this study demonstrated that there is a HERG and STAT1 interaction in breast cancer. HERG tyrosine phosphorylation by *src* tyrosine kinase can facilitate protein-protein interaction with proteins that have the *src* homology 2 (SH2) domain, which binds to phospho-tyrosine enriched protein substrates [143]. Therefore, we hypothesized that there exists an interaction between HERG and STAT1 which can be up-regulated by estrogen stimulation of *src* activity. Indeed, our data revealed novel STAT1-HERG, STAT1-*src*, and *src*-HERG interactions in cancerous breast tissue and these interactions were reinforced with estrogen stimulation in ER+ human breast cancer cell lines. Furthermore, our biotinylation results showed an increased surface expression of HERG channel upon estrogen stimulation in ER+ breast cancer cells and this upregulation was inhibited by STAT1, *src* and estrogen receptor inhibitors. Thus, STAT1 and HERG appear to exist in the same signalling complex as *src* (and possibly estrogen receptors) that are downstream of estrogen stimulation. This finding is relevant to oncogenesis, since increased estrogen level in hormone replacement therapies is associated with development of breast tumour [3]. Moreover, these novel protein-protein interactions between HERG and intracellular signalling proteins provide a mechanism for how estrogen increases proliferation in ER+ breast cancer. Future studies are needed to gain further insight into the precise interactions between HERG and signalling proteins, specifically whether the interactions are mediated by direct or indirect protein-protein interactions and to determine the precise locations in the HERG channel where these interactions are occurring.

This study demonstrates the importance of estrogen-induced HERG and STAT1 interaction in upregulation of HERG channels. However, the precise role of HERG and STAT1 interaction in overall HERG expression and cell proliferation and cancer progression needs further elucidation. Some of the possible roles of HERG-STAT1 interaction on HERG regulation can be derived from looking at where the interaction is happening. Our confocal imaging data showed that the HERG and STAT1 interaction occurred in different subcellular locations, including plasma membranes, intra-nuclear and cytosolic regions. It is known that the HERG gene has a promoter region which binds to transcription factors, such as SP1 and NF-kB [79]. It is plausible that STAT1 could also bind to the HERG gene in the same manner to regulate HERG transcription, but future studies involving chromatin immunoprecipitation assays are required to start to unravel the possible transcriptional regulation of HERG by STAT1.

Although there were some HERG and STAT1 co-labelled punctate staining observed within the nucleus of breast tumor cells as well as human breast cancer cell lines, the majority of the interactions were found at or near the plasma membranes and in the perinuclear and cytosolic regions. HERG is synthesized in the endoplasmic reticulum, and is then modified in the Golgi apparatus before being transported to the cell surface [71]. There are chaperone proteins such as Hsp70 and Hsp90 which transiently bind to HERG in the cytosol to facilitate HERG trafficking to the cell surface [144]. Since STAT1 inhibition prevented estrogen-induced plasma membrane HERG upregulation as shown from biotinylation studies, it is possible that STAT1 may play a role in HERG trafficking as well.

Among all the voltage-gated potassium channels, HERG channels are unique in that their biophysical properties are such that membrane depolarization forces these channels to undergo rapid inactivation (resulting in minimum HERG conductance) and then produce significantly more

currents upon recovery from inactivation during membrane hyperpolarization [53, 84, 119]. In contrast to most potassium channels, the overexpression of HERG channels are associated with more depolarized membrane potentials which is a typical characteristic of highly proliferating cancerous cells. We predict that after estrogen stimulation, the resting membrane potentials of ER+ human breast cancer cells will be even more depolarized compared to non-treated cells, which would be expected to contribute to the observed increase in cell proliferation. However, further electrophysiological studies are required to confirm whether membrane potentials are indeed more depolarized after estrogen stimulation and subsequent upregulation of HERG-STAT1 interaction and plasma membrane HERG expression. It is possible that STAT1 binding to HERG may play a role in regulating HERG channel conductances. N-terminal truncation of HERG channels results in much faster deactivation rates than the full-length wild type channels [145, 146]. The N terminus of HERG, which is important for slowing deactivation rate [145, 147], also has sites for tyrosine phosphorylation, which may facilitate STAT1 binding via its SH2 domain. If the STAT1 binds to HERG at its N-terminus, STAT1 may create a steric hindrance to further slow down deactivation of HERG channel. As a result, HERG conductance may increase favouring proliferation of cancer cells. However, future studies are required to establish which HERG N-terminal tyrosine residues (Y54SRA, Y329RTI, and/or Y405SPF) are tyrosine phosphorylated, and whether *src* tyrosine kinase is intimately involved in this HERG tyrosine phosphorylation. Also, it will be important to establish whether site directed mutagenesis of these tyrosine residues can abrogate the STAT1 binding, presumably resulting from the removal of the SH2 domain target substrates in HERG N-terminus. Moreover, other HERG tyrosine residues located in the S4-S5 linker (Y544SEY) or in the C-terminus (Y814ARP, Y829CDL, Y847PEF, Y1013QEL, or Y1082SAV) will need to be examined for their potential binding to the STAT1 SH2 domain. Finally, the specificity of the

STAT1 SH2 domain will also need to be tested against similar SH2 domains from other signalling molecules, including *src*-family tyrosine kinases, Grb2, Cbl and other STAT-family members, for their ability to bind to these HERG phosphotyrosine residues. To our knowledge, this is the first report of a potential STAT1 SH2 domain binding to HERG, which can have important implications in anti-cancer therapeutic development.

5. GENERAL DISCUSSION

ER positive breast cancer is the most prevalent subtype of breast cancer [5]. Currently, there are strategies for targeting estrogen signaling and ERs using anti-estrogen drugs such as tamoxifen and raloxifene [148]. Though anti-estrogen therapy has been effective in many cases, there are a number of issues with the existing anti-estrogen therapy. First, patients develop resistance to long-term use of anti-estrogen therapy. Second, anti-estrogen drugs are associated with increased risk for endometrial cancer [149]. Therefore, there is a crucial need for development of new anti-cancer therapeutics.

HERG has a great therapeutic value as an anti-cancer target as its overexpression in cancerous tissues plays a role in aberrant cell proliferation [68]. However, it is difficult to target the HERG channels that are only located in cancerous tissues. Malfunction of HERG channels in other normal tissues, such as cardiac tissue, can cause long QT syndrome type 2 [56]. HERG is also expressed in brain, gut, hematopoietic, immune and neuroendocrine cells [67, 150-152], so blocking the HERG channels directly could have adverse consequences in normal physiological functions. Therefore, molecular mechanism of HERG overexpression in cancerous cells warrants further investigation.

In the present study, we show for the first time that there is a physical complex consisting of HERG and STAT1, and that stimulation by estrogen in ER positive breast cancer leads to increased expression of both HERG and STAT1, and consequently increased interactions in different locations within a cancerous cell. The *src* tyrosine kinase is also a component of this multi-protein complex, and together with STAT1, *src* tyrosine kinase may increase the overall expression of HERG in cancerous cells. This post-translational modification of HERG by

STAT1 (and *src*) may also increase the overall function of HERG channels by altering the biophysical properties of this channel, but further electrophysiological studies are needed to test this possibility. In addition to the post-translational modification of HERG by STAT1, a transcriptional regulation of HERG by STAT1 cannot be ruled out. We found that both STAT1 and HERG colocalized inside the nucleus of both human breast cancer tumors and breast cancer cell lines. As there has been a precedence for transcription factors playing a role in regulating HERG transcription [73], it is reasonable to speculate a similar potential role for STAT1 binding to HERG promoter sequence and inducing HERG transcription. Other potential mechanisms for estrogen-induced increase in HERG surface expression (and function) include upregulation of regulatory processes involved in the forward trafficking of HERG channels to the plasma membrane, decreased endocytosis of HERG channels, increased stability of HERG channels by other post-translational modification (e.g., N-linked glycosylation), and possibly increased localization of HERG channels in specialized membrane domains (e.g., lipid rafts). Clearly, there are numerous mechanisms that could contribute to HERG overexpression in ER positive breast cancers, but our finding that HERG and STAT1 interaction appears to be specific in cancerous tissue and cancerous cells is clinically significant, as we have shown that selective disruption of this interaction with a peptide mimetic can prevent the estrogen-induced HERG overexpression and reduce cancer cell proliferation. Unlike the previous proposed therapeutic interventions based on HERG [68, 153], targeting HERG and STAT1 interaction selectively can provide a way to avoid proarrhythmic and cardiotoxic risks of targeting HERG channels. By specifically targeting the HERG-STAT1 interaction using our FR-peptide, we demonstrated that we can prevent the proliferation of ER positive breast cancer cells. Future studies are planned to study the effects of this peptide on HERG channel expression in human induced pluripotent stem cell-

derived cardiomyocytes from commercial sources, to rule out any potential cardiotoxicity side effects of this peptide.

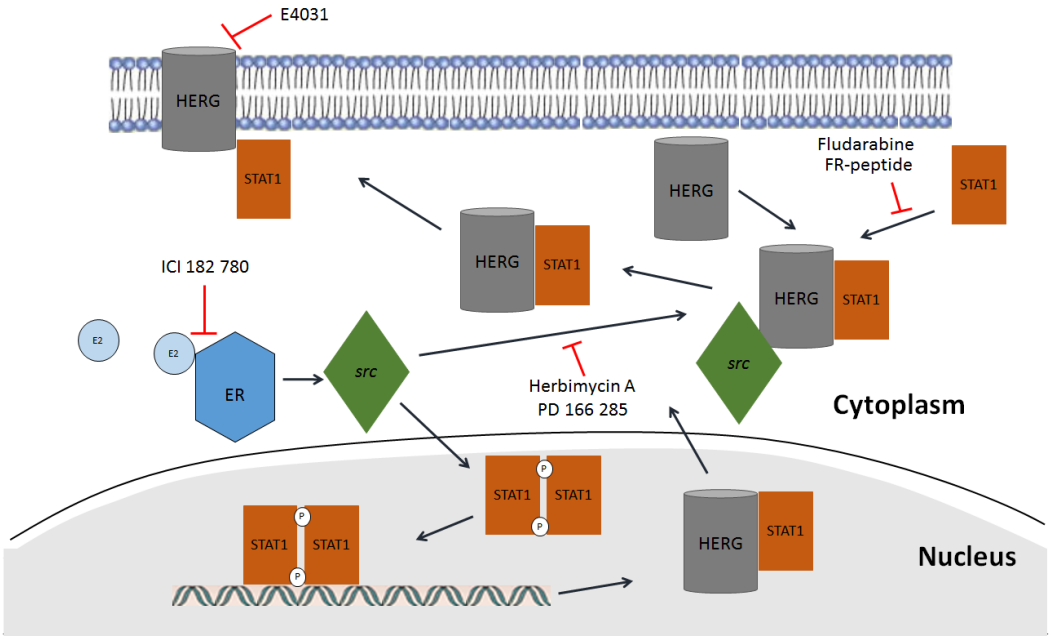


Figure 5.1. Summary of the proposed post-translational signalling pathway examined in this thesis. Black arrows indicate the direction of a downstream pathway. Red arrows indicate the drug inhibitors used in this study.

6. CONCLUSION AND FUTURE DIRECTIONS

6.1 Future directions

This study mainly focused on the post-translational modification of HERG channels. However, further investigation on transcriptional regulation of HERG is needed for better understanding of HERG regulation by transcription factor STAT1. There are several tumour suppressors (e.g. NKx3.1) and oncoproteins (e.g. Sp1 and NF-κB) which are known to regulate HERG expression by binding to the promoter region of HERG gene [79]. Similarly, STAT1 may also bind to the promoter region of HERG gene in the nucleus to regulate HERG transcription. In silico analysis can be done to determine if HERG promoter region has potential binding site for STAT1. Then chromatin immunoprecipitation can be performed to examine if STAT1 protein is binding to promoter sequence of HERG gene.

Also, further studies on HERG current conductance need to be performed in the future using FR-peptide. In the present study, we demonstrated that FR-peptide treatment results in reduced number of surface HERG channels. However, it is important to examine the effect of FR-peptide on HERG current conductance to investigate the functional consequences of disrupting the STAT1-HERG interaction at the plasma membrane and the overall resting membrane potential of human breast cancer cells.

To move these findings to clinical translation, it would be valuable to establish in an animal breast cancer model whether the FR-peptide is effective *in vivo*. It is known that breast tumour cells can metastasize to other organs, including the lungs, bone and brain [154-157]. In collaboration with Dr. Mendez's lab (Department of Surgery, University of Saskatchewan), the Cayabyab lab is currently developing a rat model of breast cancer brain metastasis, whereby ER+ MCF-7 cancer cells are transplanted into the rat brain striatum. Similar procedures from

Mendez's lab are routinely used to transplant stem cells for Parkinson's disease therapy. Rats are administered with daily doses of cyclosporine A after MCF-7 transplantation in order to prevent xenograft rejection. In addition, the FR-peptide and its scrambled versions are injected intraperitoneally for 4 weeks post-transplantation. The pilot studies show that the growth of MCF-7 cells was greatly blunted by FR-peptide but not by the scrambled version. This suggests the cell-permeable FR-peptide successfully penetrated the blood brain barrier and inhibited HERG-STAT1 interaction *in vivo*. Future studies are required to confirm these pilot studies and to further characterize the effects of FR-peptide on the transplanted cells and normal brain cells (e.g., neurons, astrocytes, microglia, and oligodendrocytes) at the transplanted brain region. It will also be important in future studies to determine whether ER- cells (e.g., MDA-MB-231, BT-20, SK-BR3) can also be successfully transplanted and their growth inhibited by the FR-peptide. Since these ER- breast cancer cell lines are considered to be more aggressive than ER+ breast cancer cell lines, it will be important to demonstrate whether FR-peptide will have greater efficacy in preventing breast cancer cell growth *in vivo*.

6.2 Conclusion

This thesis examined the underlying mechanism of HERG overexpression in ER positive breast cancer using confocal microscopy, cell proliferation assay and different biochemistry techniques. The data presented in this study show for the first time that (i) HERG and STAT1 interact in breast cancerous cells and tissues but not in normal brain or heart tissues; (ii) estrogen increases HERG and STAT1 interactions; (iii) estrogen upregulates surface HERG channels by increasing HERG and STAT1 interactions; (iv) FR-peptide can prevent estrogen induced cell proliferation by disrupting HERG and STAT1 interactions. This study not only elucidates the molecular mechanism for HERG overexpression in ER positive human breast cancer but also

provides a method to prevent the overexpression. The new findings in this study may open up a new area for treating breast cancer.

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